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Award Number: DAMD17-01-1-0779

TITLE: Neuroprotective Ganglioside Derivatives

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REPORT DATE: September 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

20041215 032

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2004	3. REPORT TYPE AND DATES COVERED Annual (15 Aug 2003 - 14 Aug 2004)	

4. TITLE AND SUBTITLE
Neuroprotective Ganglioside Derivatives

5. FUNDING NUMBERS
DAMD17-01-1-0779

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words) In this study, neuroprotective ganglioside derivatives are studied in an attempt to devise neuroprotective agents targeted to specific points in cell death pathways. GM1 ganglioside and several of its chemically modified derivatives are neuroprotective in a variety of neurotoxic models. Here, ganglioside functional groups required for neuroprotection and blood-brain barrier (BBB) permeance are determined. Cell death mechanisms are also defined, as are the mechanism(s) by which ganglioside derivatives intervene in the cell death process. In the third year, syntheses of various ganglioside derivatives have been accomplished. A significant delay in the proposed work was encountered, however, when a commercial reagent was acidic beyond specifications and, therefore, decomposed the lysoGM1 derivative that is also the starting material for many of the other derivatives. Negotiations are currently underway to determine company culpability and fair replacement. Fatty acid derivatives of GM1 have been tested in the SH-SY5Y cell culture system. LysoGM1, LIGA 20, N-butylGM1, N-MyristylGM1, and GM1 inner ester were all cytoprotective but at different effective concentrations. AsialoGM1 was not cytoprotective.

14. SUBJECT TERMS Ganglioside derivatives, SH-SY5Y cells	15. NUMBER OF PAGES 54
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

Gangliosides are naturally occurring acidic glycolipids that are enriched in neural tissue. They broadly protect different types of neurons *in vitro* (1;2) and preserve diverse neuronal (3-5) and behavioral (6;6) functions from a variety of neurotoxic insults *in vivo*. The fact that gangliosides appear to protect neurons from various neurotoxic insults by several different mechanisms makes them ideal candidates for, or models upon which to derive, therapeutic agents for Parkinson's disease and other neurodegenerative diseases with potentially diverse pathological mechanisms. Gangliosides are heterogeneous compounds differing in the composition of their lipophilic and hydrophilic moieties. It has been shown with the monosialoganglioside GM1 that the cytoprotective effects and blood-brain barrier permeance differ among the various molecular structures. This implies that the protective effects of gangliosides can be optimized by semisynthetically varying their lipophilic and hydrophilic moieties. Many neurotoxins work through mechanisms that involve mitochondrial electron transport chain inhibition and oxidative stress, proteasomal inhibition, or excitotoxicity. Gangliosides have been shown to be protective against a range of neurotoxins in various models. For example, GM1 protects dopaminergic neurons from MPTP (7;8) and MPP$^+$ (9) (a mitochondrial complex I inhibitor) even when administered after neurotoxin exposure (10). Gangliosides appear to be protective through several mechanisms. These minimally include their effects on growth factor production and receptor activation (11), signal transduction (12), membrane order and raft formation (13), calcium homeostasis (14), membrane trafficking, excitatory processes (15), altered enzyme activity (such as superoxide dismutase (16), catalase (17) or phosphatidylinositol 3-kinase (2)) and modified protein kinase C translocation (3). It is, of course, important to ultimately understand the mechanism of ganglioside neuroprotection. There is, however, neither an adequate understanding of ganglioside functional groups and required structure necessary to produce significant neuroprotection, nor a sufficient knowledge of the functional group alterations that allow ganglioside derivatives to effectively cross the BBB while maintaining cytoprotective ability. In fact, investigations into ganglioside protective mechanisms are daunting endeavors because of the apparent diverse modes of action. Thus, one approach to defining structure-function relationships in ganglioside neuroprotection is to first describe the structural requirements for neuroprotection and then to define the mechanisms of action.

One problem in using gangliosides for therapeutic purposes is that they are ionic and hydrophilic. This prevents them from efficiently crossing the BBB. An approach to overcoming the BBB limitation is to make gangliosides more lipophilic by derivatizing the parent compounds (semisynthetic gangliosides), and, presumably, making them better able to cross the BBB. The internal ester (lactone) of GM1 is chemically neutral (and, therefore, more lipophilic) and it is also neuroprotective *in vivo*,

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often more potently than the parent GM1. ([19](19)), ([20](20)), ([21](21)), a ([22](22)). The apparent improvement, compared to the parent GM1, in the *in vivo* neuroprotection by ganglioside derivatives such as the internal ester or LIGA 20, may be due to either or both improved neuroprotective properties, and improved ability to cross the BBB.

This study examines ganglioside functional group derivatives that provide cytoprotection AND effectively cross the BBB; information that will provide a basis for future studies of neuroprotective mechanisms. This research studies the ability of ganglioside derivatives to be cytoprotective in *in vitro* models using the dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium (MPP^+) and the SH-SY5Y human neuroblastoma cell line. Derivatives determined to have therapeutic potential are tested *in vitro* for their ability to cross a brain capillary endothelial cell culture model of the BBB. Derivatives that are both cytoprotective and that effectively cross the *in vitro* BBB model will be tested *in vivo* for their ability to neuroprotect dopaminergic neurons in both chronic and acute neurotoxicity models using the MPP^+ precursor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This research studies the hypothesis that "changes in ganglioside ceramide and/or oligosaccharide functional groups will improve neuroprotection through changes in cytoprotection and BBB transcytosis." This research will provide a basis to improve ganglioside neuroprotection in neurodegenerative diseases, e.g., Parkinson's disease and neurotoxin exposure.

BODY

Response to reviewer's comments on second annual report

(1) Objective #1 – *In progress, slow, but some success reported.......no data for quantitative yield or structural determination, such as chemical and mass spectrometry, as originally planned.....-* As described below, this project has been impeded by the substantial loss of lysoGM1 due to a commercial solvent that was acidic well beyond specifications. Considerable time and effort is required to produce this material from GM1. It is then used to synthesize the fatty acid molecular species used for *in vitro* testing and for the production of radiolabeled gangliosides. It is extremely unstable in an acidic environment. Negotiations are in progress with the manufacturer to recoup losses.

Still, several derivatives have been synthesized and characterized (Table 1). Gangliosides and their derivatives were characterized by chromatographic behavior compared to ganglioside standards, chemical reaction (e.g., hydrolysis of esters to the parent compounds), colorometric responses to specific sprays on HPTLC (e.g., resorcinol for sialic acid; orcinol for sugars, and ninhydrin for free amines) and by MS and MS/MS instrumental analyses. MS and MS/MS spectra of the derivatives were acquired by infusion of methanol solutions of each compound at 5 ul/min directly into the electrospray

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ionization source of a Finnigan LCQ quadrupole ion trap mass spectrometer equipped with a Finnigan Xcalibur data system. Negative ion electrospray ionization was performed with 4,500 V source potential and MS/MS spectra were obtained using 33% collisional activation energy. The derivatives were identified by the [M-H]⁻ ion and MS/MS fragment ions. Characterization of several inner esters of N-acylGM1 derivatives is in process.

Compound	Resorcinol	Orcinol	Ninhydrin	Retained by Ion Exchange	[M-H]⁻ C18:0-d18:1	[M-H]⁻ C18:0-d20:1
GM1	+	+	-	yes	1544	1572
GM1 inner ester	+	+	-	no	1526	1554
lysoGM1	+	+	+	untested	1261	1289
N-acetyl GM1 (LIGA4, C2)	+	+	-	yes	1320	1348
N-dichloroacetylGM1 (LIGA20, dichloroC2)	+	+	-	untested	1388	1416
GM1 gangliosidol	+(faint)	+	-	no	1530	1558
N-acetyl GM1 inner ester	+	+	-	no	1302	1330
AsialoGM1	-	+	-	no	1253	1281
N-butyl GM1 (C4)	+	+	-	yes	1348	1376
N-myristyl GM1 (C14)	+	+	-	yes	1488	1516
N-arachidyl GM1 (C20)	+	+	-	yes	1572	1600
N-cerotyl GM1 (C26)	+	+	-	yes	1656	1684

Table 1. Characterization of gangliosides and ganglioside derivatives. C18:0, 18 carbon saturated fatty acid; d18:1, sphingosine; d20:1,eicosasphingosine (20 carbons, 1 double bond).

(2) Objective #2 *Although the work is in progress, it is slow and at risk of being diffuse and poorly focused...... trafficking studies not very satisfying.........nothing on dopamine transporter* – At the reviewer's suggestion, we limited, for the results reported here, the assay of viability to the MTT assessment.

The intracellular trafficking of exogenous ganglioside derivatives is being approached in collaboration with Dr. Wayne Lencer and Dr. Anne Wolf, Childrens Hospital, Boston, MA. These investigators are utilizing a glioma cell line for their studies. They have found that, indeed, the plasma membrane uptake kinetics and cholera toxin binding of the short chain ceramide fatty acid derivative (N-butyl GM1) is significantly different than that for the parent compound. Conditions for the uptake kinetics and subcellular trafficking are being defined in the glioma cell line and will then be applied to the SH-SY5Y cells. This approach is being taken in the interest of efficient allocation of time and resources.

Further, we are aware of the role that the dopamine transporter plays in MPP+ toxicity. The transporter is present in the SH-SY5Y cells but we have no evidence that the toxin alters its gene expression.

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(3) Objective #3 and Objective #4 *Considered as not yet addressed* – These objectives have not been addressed. The loss of our lysoGM1 seriously delayed synthesis of the radiolabled ganglioside derivatives needed for the BBB studies. The endothelial cell model of the BBB is now functional and the studies on BBB permeance will be initiated upon acquisition of the necessary labeled compounds. We anticipate these derivatives will be available within the next month barring unforeseen circumstances. Objective #4 will be initiated immediately upon identification of an effective cytoprotective derivative that crosses the BBB model effectively.

Report

This proposal consists of 4 specific objectives in the **Statement of Work**.

Statement of Work, Objective 1- Semisynthetic ganglioside derivatives will be synthesized from gangliosides isolated and characterized in the P.I.'s laboratory. These will include derivatives of the ceramide fatty acids, oligosaccharide functional groups, including internal esters, asialo, and reduced carboxylic acid (gangliosidol), and combinations of ceramide and oligosaccharide derivatives. Syntheses will be performed as described in Methods. Semisynthetic ganglioside derivatives will be characterized by chemical and mass spectrometric techniques.

Techniques for the synthesis of GM1 derivatives are now well established in this laboratory. In this reporting period, one serious problem arose that resulted in the destruction of the total lysoGM1 supply that had been synthesized in this project. LysoGM1 is the starting material for all of the GM1 fatty acid derivatives, both unlabeled and radiolabled. This compound is sensitive to acid hydrolysis and must be handled in a neutral or slightly alkaline condition. Chloroform purchased from a reputable source (Fisher Scientific) contained acid well beyond product specifications. This problem was not discovered for some time because the visual consequences manifested themselves identically to problems with chromatographic purification. This source of chloroform has been used in this laboratory for two decades with no previous problems. When replacement chloroform was ordered, the acidic condition still existed even though the company was aware of the problem and even though it had assured us the problem was resolved. Currently, we are negotiating with Fisher Scientific to obtain compensation for the lost time, materials, and starting material.

Statement of Work, Object 2 - Semisynthetic derivatives that specifically retain or improve the cytoprotective properties of the parent compound will be determined by testing them *in vitro* using an MPP^+ model of neurotoxicity in SH-SY5Y cells. Cytoprotection will be evaluated by use of the MTT assay, trypan blue exclusion with cell counting, and neurochemical analysis of DA, DOPAC, and HVA.

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The ability of GM1 (no preincubation) to protect RA-differentiated SH-SY5Y cells from MPP$^+$ toxicity was determined with the MTT assay 48 hr after toxin exposure (Figure 1). The MTT assay provides an evaluation of mitochondrial status. Experiments consisted of at least 4 wells per data point and the experiment was performed in triplicate.
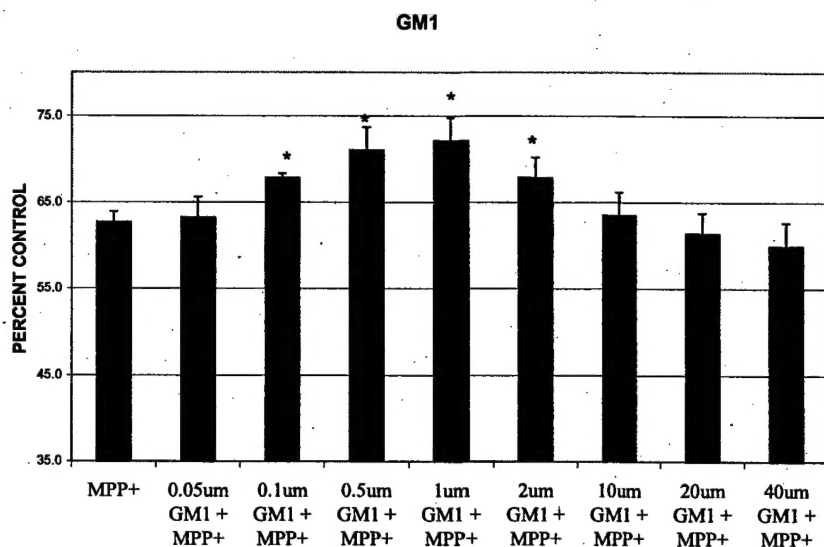

Figure 1. GM1 cytoprotection. Increasing concentrations of GM1 partially protected SH-SY5Y cells from MPP$^+$ (1mM) toxicity. Concentrations at or above 2uM did not provide protection.
Values represent the mean $\pm$ SEM of 4 wells
* p<0.05

GM1 provided partial protection against MPP$^+$ toxicity with maximal protection at 1 uM. Higher GM1 concentrations were not protective. The mechanism of this loss is unknown but may be due to the detergent effect of the amphipathic GM1. This phenomenon was seen with all of the derivatives tested and will earn further investigation in another project. Incubation of GM1 alone with SH-SY5Y cells under these conditions did not significantly alter cell viability compared to no GM1 controls.

The literature indicates that gangliosides may require a preincubation period prior to addition of the toxin. We tested the need for preincubation with the parent GM1. The SH-SY5Y cells were tested at 48 h after MPP+ exposure. The cells were preincubated with GM1 for 2 hr before toxin addition and

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the amount of protection was compared to that with no preincubation and to the presence of toxin alone (Figure 2). Preincubation did not improve protection. Shorter (0.25 hr and 0.5 hr) and longer (4 hr and 6 hr) incubation times were also examined in a separate experiment with no improvement in cytoprotection compared to no preincubation controls. Therefore, for the MPP$^+$ experiments, no preincubation of GM1 or the derivatives was used.
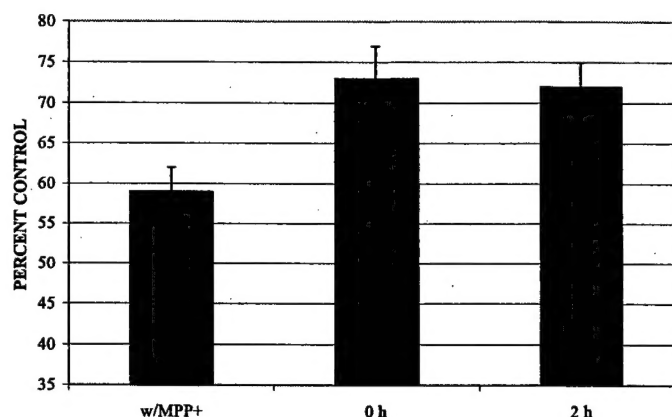

Figure 2. Preincubation of the GM1 is not required for cytoprotection.
Values are mean ± SEM
* different from MPP$^+$ alone, p<0.01, N = 6 wells

Although preincubation is not required for GM1 in this experimental paradigm, preincubation experiments to investigate optimal preincubation intervals for each new derivative to be tested are included in the experimental design.

Subsequent to establishing conditions for GM1, several GM1 derivatives have been tested *in vitro* using the MTT assay. For each derivative tested, dose response and preincubation curves were run. Cell culture plates (48-well) were utilized and each data point consisted of either 4 or 6 wells depending on the experimental design and each experiment was run in duplicate or triplicate with the

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exception of the GM1 inner ester which has been run only once to this time. For brevity, comparisons are presented at the derivative concentration with maximal cytoprotection (Figure 3). All GM1 derivatives were cytoprotective with the exception of the asialo derivative, implying that the sialic acid is an important constituent for cytoprotection. LysoGM1, N-butylGM1, and N-myristylGM1 cytoprotect comparable to GM1 but their effective concentrations are significantly less than that for GM1 (lysoGM1 0.1 µM; butylGM1 0.05 uM; N-myristylGM1 0.04 uM). LysoGM1 (no ceramide fatty acid) was unexpectedly protective. This could imply that the fatty acid is not important in the cytoprotective mechanism or that the cytoprotective mechanism is
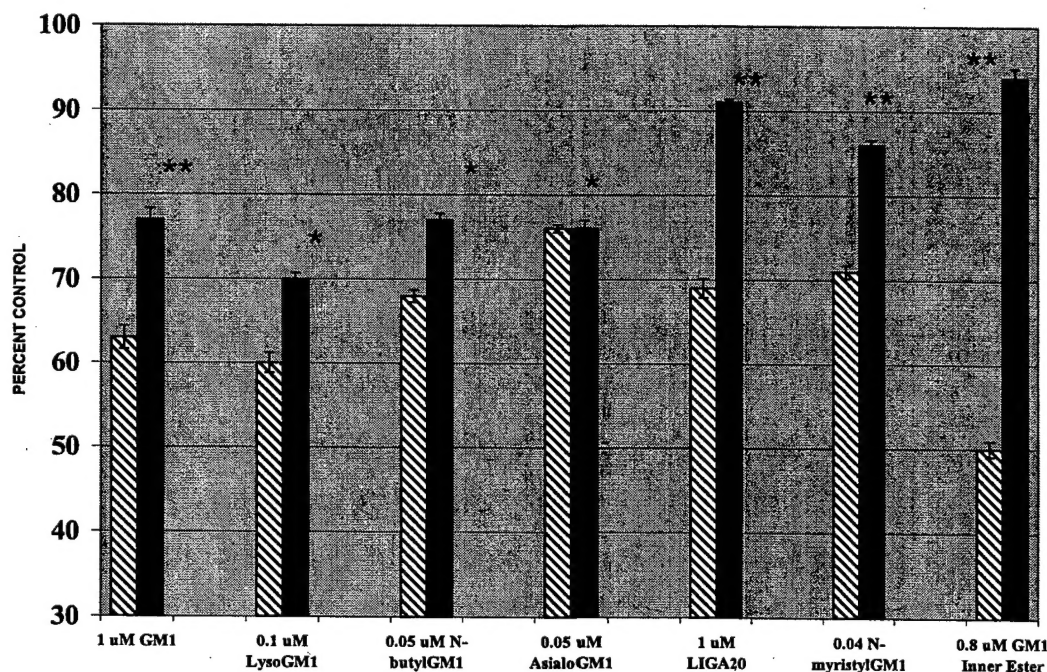

Figure 3. Comparisons of GM1 derivative cytoprotection. Values are expressed as percent control $\pm$ SEM. Stripped columns are percent control of MPP[+]-treated RA-differentiated SH-SY5Y cells. Filled columns are percent control $\pm$ SEM of MPP[+]-treated cells in the presence of GM1 or GM1 derivative. Differences between MPP[+] with or without GM1 or GM1 derivatives are significant except for asialoGM1.
*p<0.05
**p,0.01

different for lysoGM1. The likelihood of different mechanisms of action is supported by the fact that different chain length ceramide fatty acid gangliosides differentially regulate growth factor signals

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(23). Also, the ceramide fatty acid is apparently important in the determination of BBB permeance. Experiments with combinations of derivatives will be tested for any additive effects of separate mechanisms. The protection afforded by the GM1 internal ester appears to be the most potent. Internal esters of the other derivatives are being prepared. Considerations for *in vivo* testing will include blood-brain barrier permeance and effective concentration rankings.

Additional experiments have been performed (in collaboration with Dr. Kelly Conn, Dr. John Wells, Dr. Richard Fine at the VA Hospital, Bedford, MA) to further establish the mechanism of MPP^+ toxicity, which will help in the delineation of mechanisms by which ganglioside derivatives are cytoprotective. To further define MPP^+ toxic mechanisms and Parkinson's disease neuronal degeneration, RT-PCR analysis was used to characterize the transcriptional response of retinoic acid-differentiated SH-SY5Y cells to MPP^+ exposure. We have shown that the protein disulfide isomerase (PDI) family member pancreatic protein disulfide isomerase (PDIp), previously considered exclusively expressed in pancreatic tissue, is uniquely upregulated among PDI family members within 24 hours following exposure of retinoic acid-differentiated SH-SY5Y cells to 1 mM MPP^+ or to 10uM of the proteasomal inhibitor lactacystin (Appendix, Item #1). Further, lactacystin treatment increases clusterin expression. Clusterin (apolipoprotein J) is a highly conserved, multifunctional vertebrate glycoprotein. Several isoforms exist and the predominant secreted isoform (sCLU) is associated with cell death and binds a variety of partly unfolded, stressed proteins including those associated with Lewy bodies in Parkinson's disease (Appendix, Item #2). The development of familial and sporadic Parkinson's disease has been associated with the ubiquitin-proteasome system dysfunction and aberrant protein degradation. These findings on altered gene expression in experimental models of Parkinson's disease provide insight into possible protective mechanisms.

In a collaborative study with Dr. Wayne Lencer and Dr. Anne wolf, we have been looking at exogenous ganglioside trafficking as suggested by a reviewer of the previous report. Initial studies have been performed in a glioma cell line. Preliminary results indicate that the shorter chain

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ganglioside (N-butylGM1) associates more rapidly with the plasma membrane than does the parent GM1 but that the parent GM1 is a more effective receptor for cholera toxin subunit than the butyl derivative. These experiments are being repeated and the intracellular target for the bound cholera toxin is now under investigation. When conditions are well established, the uptake kinetics and intracellular trafficking will be examined in the SH-SY5Y cells.

Statement of Work, Objective 3, <u>Effective cytoprotective semisynthetic ganglioside derivatives that effectively cross a brain capillary endothelial cell model of the blood-brain barrier (BBB) will be determined. Model BBB transcytosis will be assessed by liquid scintillation counting of radiolabeled derivatives in aliquots taken from the lower wells of Transwell cell culture plates.</u> These studies have been delayed because of the lysoGM1 loss described above. Radiolabeled derivatives are now being synthesized using galactose oxidase and [^{3}H]-sodiumborohydride and the experiments will soon begin.

Statement of Work, Objective 4, <u>Semisynthetic ganglioside derivatives that effectively protect neurons *in vivo* using chronic and acute MPTP administration models of neurotoxin insult will be determined by testing, in mice, those derivatives that both cytoprotect the SH-SY5Y cells from MPP$^+$ toxicity *in vitro* AND that effectively cross the *in vitro* BBB model. The chronic and acute models represent apoptotic and necrotic cell death mechanisms, respectively. Neuroprotection will be evaluated by neurochemical analysis of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), and by neuronal counts of Nissl substance, tyrosine hydroxylase (TH) and dopamine transporter (DAT) positive neurons of the substantia nigra pars compacta.</u> Work on this objective is also delayed but will begin upon completion of the BBB studies.

KEY RESEARCH ACCOMPLISHMENTS

- Increasing concentrations of GM1 partially protects SH-SY5Y cells from MPP$^+$ (1mM) toxicity. Concentrations at or above 2uM appeared to make the cells more vulnerable to MPP$^+$ toxicity.
- Preincubation of the GM1 is not required for cytoprotection
- Ganglioside derivatives provide cytoprotection comparable to the parent GM1 but at lower concentrations
- GM1 protects against low potassium induced apoptotic cell death
- MPP$^+$ or lactacystin upregulate the expression of PDIp
- Lactacystin increases clusterin expression

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REPORTABLE OUTCOMES

Manuscripts

Conn,K., Ullman MD, Larned MJ, Eisenhauer PB, Fine RE, Wells JM: cDNA Microarray Analysis of Changes in Gene Expression Associated with MPP$^+$ Toxicity in SH-SY5Y Cells, *Neurochem. Res.* Neurochemical Research (2003) 28: 1873-1881

Conn KJ, Gao W, McKee A, Lan MS, Ullman MD, Eisenhauer PB, Fine RE and Wells JM. Identification of the Protein Disulfide Isomerase Family Member PDIp in Experimental Parkinson's Disease and Lewy Body Pathology. Brain Research. In press.

Carreras I, Garrett-Young R, Ullman MD, Eisenhauer PB, Fine RE, Wells JM, Conn KJ. Upregulation of clusterin/apolipoprotein J in Lactacystin-treated SH-SY5Y Cells. J. Neurosci. Res. Submitted.

Abstracts

Conn KJ, Garrett-Young R, Ullman MD, Gao WW, Eisenhauer PB, Fine RE, Wells JM. Upregulation of Clusterin/apolipoprotein J in Lactacystin-treated SH-SY5Y Cells. 9th International Conference on Alzheimer's Disease and Related Disorders, July 17-22, 2004

Conn KJ, Carreras I, Ullman MD, Eisenhauer PB, Fine RE, Wells JM. Upregulation of Growth/Differentiation Factor-15 (GDF-15)/Macrophage Inflammatory Peptide-1 (MIC-1) by MPP$^+$ and Lactacystin in differentiated SH-SY5Y Cells. Society for Neuroscience, 34th Meeting, San Diego, CA 2004

Presentations

Ullman MD. Neuroprotective Ganglisode Derivatives: An Overview. Department of Health and Environmental Sciences, University of Massachusetts Lowell, Lowell, MA. April 21, 2004

CONCLUSIONS

Our experience in synthesizing and developing HPLC procedures to purify semisynthetic ganglioside derivatives provides the opportunity to find improved cytoprotective ganglioside derivatives, to define required functional groups for cytoprotection, and to initiate studies to determine cytoprotective mechanisms. Regrettably, the studies have been impeded by the unfortunate and unavoidable loss of

the lysoGM1 supply. Still, progress has been made in the *in vitro* studies. GM1, to approximately 2uM) partially protected SH-SY5Y neuroblastoma cells from MPP$^+$ toxicity. GM1 concentrations above 2uM appeared to make the cells more vulnerable to MPP$^+$ toxicity and this phenomenon warrants further investigation in another study. Preincubation of the GM1 was not required. Ganglioside derivatives provide comparable cytoprotection but at lower concentrations than that for the parent compound. These compounds will be tested in the endothelial cell model of the BBB.

Experiments have been performed to further establish the mechanism of MPP$^+$ toxicity, which will help in the delineation of mechanisms by which ganglioside derivatives are cytoprotective. Experimental modeling of Parkinson's disease neurodegeneration using MPTP and MPP$^+$ identified changes in gene expression of different endoplasmic reticulum stress proteins associated with MPTP- and Parkinson's disease-related neurodegeneration. The protein disulfide isomerase (PDI) family member pancreatic protein disulfide isomerase (PDIp) was found to be uniquely upregulated among PDI family members following exposure of retinoic acid-differentiated SH-SY5Y cells to MPP$^+$ or to the proteasomal inhibitor lactacystin. Further, lactacystin treatment increased clusterin expression. The development of familial and sporadic Parkinson's disease has been associated with the ubiquitin-proteasome system dysfunction and aberrant protein degradation. These findings on altered gene expression in experimental models of Parkinson's disease emphasize the importance of understanding the mechanisms of MPP$^+$ toxicity in attempts to further define mechanisms of ganglioside neuroprotection because by better understanding neurotoxic and neuroprotective mechanisms, specific steps in the cell death process can be targeted.

REFERENCES

1. Jonsson G, Gorio A, Hallman H, Janigro D, Kojima H, Zanoni R. Effect of GM1 ganglioside on neonatally neurotoxin induced degeneration of serotonin neurons in the rat brain. Develop Brain Res 1984;16:171-80.

2. Ryu, B. R., Choi, D. W., Hartley, D. M., Costa, E., Jou, I., and Gwag, B. J. Attenuation of cortical neuronal apoptosis by gangliosides. Journal of Pharmacology and Experimental Therapeutics 290, 811-816. 1999.

3. Kikuchi S, Kim SU. Glutamate neurotoxicity in mesencephalic dopaminergic neurons in culture. J Neurosci Res 1993;36:558-69.

4. Aguis L, Hadjiconstantinou M, Qu Z-X, Neff NH, Pearl DK, Yates AJ. GM1 ganglioside and darkly staining neurons in brains of rats subjected to neonatal hypoxia-ischemia. Int J Dev Neurosci 1994;12:623-30.

5. Bianchi R, Janigro D, Milan F, Giudici G, Gorio A. In vivo treatment with GM1 prevents the rapid decay of ATPase activities and mitochondrial damage in hippocampal slices. Brain Res 1986;364:400-4.

6. Silva RH, Bergamo M, Vital BMBF, Frussa R. Effects of neonatal GM1 administration on the discriminative avoidance behavior of adult rats. Ann N Y Acad Sci 1998;845:425.

7. Weihnuller, F. B., Hadjiconstantinou, M., Bruno, J. P., and Neff, N. H. Administration of GM1 ganglioside eliminates neuroleptic-induced sensorimotor deficits in MPTP-treated mice. Neuroscience Letters 92, 207-212. 1988.

8. Weihnuller, F. B., Hadjiconstantinou, M., Bruno, J. P., and Neff, N. H. Continued administration of GM1 ganglioside is required to maintain recovery from neuroleptic-induced sensorimotor deficits in MPTP-treated mice. Life Sciences 45, 2495-2502. 1989.

9. Dalia A, Neff NH, Hadjiconstantinou M. GM1 ganglioside improves dopaminergic markers of rat mesencephalic cultures treated with MPP$^+$. J Neurosci 1993;13:3104-11.

10. Stull ND, Schneider JS, Iacovitti L. GM1 ganglioside partially rescues cultured dopaminergic neurons from MPP$^+$-induced damage: Dependence on initial damage and time of treatment. Brain Res 1994;640:308-15.

11. Svennerholm L. Gangliosides--A new therapeutic agent against stroke and Alzheimer's disease. Life Sci 1994;55:2125-34.

12. Koike T, Fehsel K, Zielasek J, Kolb H, Burkart V. Gangliosides protect from TNFα-induced apoptosis. Immunol Lett 1993;35:207-12.

13. Gniadecki R, Christoffersen N, Wulf HC. Cholesterol-rich plasma membrane domains (lipid rafts) in keratinocytes: importance in the baseline and UVA-induced generation of reactive oxygen species. J Invest Dermatol 2002;118:582-8.

14. Hoffman PL, Iorio KR, Snell LD, Tabakoff B. Attenuation of glutamate-induced neurotoxicity in chronically ethanol-exposed cerebellar granule cells by NMDA receptor antagonists and ganglioside GM$_1$. Alcoholism:Clin Exp Res 1995;19:721-6.

15. Bachis A, Rabin SJ, Del Fiacco M, Mocchetti I. Gangliosides prevent excitotoxicity through activation of TrkB receptor. Neurotox Res 2002;4:225-34.

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16. Mahadik SP, Makar TK, Murthy JN, Ortiz A, Wakhade CG, Karpiak SE. Temporal changes in superoxide dismutase, glutathione peroxide and catalase levels in primary and periischemic tissue: monosialoganglioside (GM1) treatment effect. Mol Chem Neuropath 1991;In press.

17. Fighera MR, Bonini JS, Frussa-Filho R, Dutra-Filho CS, Hagen ME, Rubin MA, Mello CF. Monosialoganglioside increases catalase activity in cerebral cortex of rats. Free Radic Res 2004;38:495-500.

18. Schneider JS, DiStefano L. Response of the damaged dopamine system to G_{M1} and semisynthetic gangliosides: Effects of dose and extent of lesion. Neuropharmacology 1995;34:489-93.

19. Dunbar GL, Lescaudron LL, Stein DG. Comparison of GM1 ganglioside, AGF2, and D-amphetamine as treatments for spatial reversal and place learning deficits following lesions of the neostriatum. Behav Brain Res 1993;54:67-79.

20. Emerich DF, Walsh TJ. Ganglioside AGF2 prevents the cognitive impairments and cholinergic cell loss following intraventricular colchicine. Experiment Neurol 1991;112:328-37.

21. Emerich DF, Walsh TJ. Ganglioside AGF2 promotes task-specific recovery and attenuates the cholinergic hypofunction induced by AF64A. Brain Res 1990;527:299-307.

22. Seren MS, Rubini R, Lazzaro A, Zanoni R, Fiori MG, Leon A. Protective effects of a monosialoganglioside derivative following transitory forebrain ischemia in rats. Stroke 1990;21:1607-12.

23. Rabin SJ, Bachis A, Mocchetti I. Gangliosides Activate Trk Receptors by Inducing the Release of Neurotrophins. J B C 2002;277:49466-72.

APPENDICES

1. Journal article

Conn KJ, Gao W, McKee A, Lan MS, **Ullman MD**, Eisenhauer PB, Fine RE, Wells JM: Identification of the protein disulfide isomerase family member PDIp in experimental and idiopathic Parkinson's disease. *Brain Research*. In press.

2. Journal Article

Carreras I, Garrett-Young R, **Ullman MD**, Eisenhauer PB, Fine RE, Wells JM, Conn KJ. Upregulation of Clusterin/Apolipoprotein J in Lactacystin-treated SH-SY5Y Cells. Submitted to *Journal of Neuroscience Research*

1

2 Research report

3 Identification of the protein disulfide isomerase family member PDIp in
4   experimental Parkinson's disease and Lewy body pathology

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14

15 **Abstract**

16   Parkinson's disease (PD) is a slowly progressing neurodegenerative disorder with no clear etiology. Pathological hallmarks of the
17 disease include the loss of dopaminergic neurons from the substantia nigra (SN) and the presence of Lewy bodies (LBs) (α-synuclein and
18 ubiquitin-positive, eosinophilic, cytoplasmic inclusions) in many of the surviving neurons. Experimental modeling of PD neurodegeneration
19 using the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methyl-4-phenyl-pyridinium (MPP^+) has identified
20 changes in gene expression of different endoplasmic reticulum (ER) stress proteins associated with MPTP- and PD-related
21 neurodegeneration. We show that the protein disulfide isomerase (PDI) family member pancreatic protein disulfide isomerase (PDIp),
22 previously considered exclusively expressed in pancreatic tissue, is uniquely upregulated among PDI family members within 24 h following
23 exposure of retinoic acid (RA)-differentiated SH-SY5Y human neuroblastoma cells to either 1 mM MPP^+ or 10 μM of the highly specific
24 proteasome inhibitor lactacystin. RT-PCR confirms PDIp expression in brain of post-mortem human PD subjects and immunohistochemical
25 studies demonstrate PDIp immunoreactivity in LBs. Collectively, these findings suggest that increased PDIp expression in dopaminergic
26 (DA) neurons might contribute to LB formation and neurodegeneration, and that this increased PDIp expression may be the result of
27 proteasome impairment.
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29 *Theme:* Disorders of the nervous system
30 *Topic:* Degenerative disease: Parkinson's

31 *Keywords:* 1-Methyl-4-phenyl-pyridinium; Parkinson's disease; Lewy body; Dementia with Lewy bodies; Pancreatic protein disulfide isomerase

32
33 ## 1. Introduction

34   Parkinson's disease (PD) afflicts about 1% of people over
35 age 65 and 4–5% of people over age 85, making it the most
36 common movement disorder and the second most common

neurodegenerative disorder in humans [21]. Because of its 37
prevalence, PD is a major societal health problem. Presently, 38
there is no cure, diagnostic marker, or clear etiology for the 39
disease. The pathological hallmarks of PD are the degen- 40
eration of dopamine (DA)-containing neurons in the 41
substantia nigra (SN) and the presence of Lewy bodies 42
(LBs) in many of the surviving DA neurons [11]. 43

    LBs are 5–25 μm hyaline intraneuronal inclusions 44
classically consisting of three layers of varying eosinophilia 45
[13]. LBs are found in neurons in several neurodegenerative 46

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47 disorders such as PD, Dementia with Lewy bodies (DLB),
48 and Alzheimer's disease (AD) [11,4]. LBs contain a variety
49 of proteins including phosphorylated neurofilament, ubiq-
50 uitin, and α-synuclein [11,29,33].

51 The majority of PD cases are sporadic; however, the
52 identification of a number of genes associated with rare
53 familial forms of PD has provided important insights into
54 the underlying mechanisms of the disease. These genes,
55 encoding α-synuclein, parkin, UCH-L1, DJ-1, and PINK1
56 have implicated protein misfolding, oxidative stress, and
57 impairment of the ubiquitin-proteasome system (UPS), in
58 the pathogenesis of the disease [14,25,4,5,35]. The ubiq-
59 uitin-proteasome system (UPS) is the primary biochemical
60 pathway responsible for the degradation of normal and
61 abnormal intracellular proteins [15,16]. Proteolysis by the
62 proteasome is specifically inhibited by the Streptomyces
63 metabolite lactacystin [9]. UPS failure leads to protein
64 accumulation and cell death [32,36].

65 The accumulation of misfolded/unfolded proteins within
66 cells triggers an endoplasmic reticulum (ER) stress response
67 [18]. Cellular changes associated with the ER stress
68 response include the unfolded-protein response (UPR).
69 The UPR is characterized by the induction of gene
70 expression of ER-localized protein-folding catalysts and
71 protein chaperones and also by the inhibition of protein
72 translation by phosphorylation of the eukaryotic initiation
73 factor-2α (eIF-2α). Under conditions of severe ER stress,
74 the apoptotic cell death pathway is activated [18].

75 We [3] and others [17,31] have recently demonstrated
76 that the UPR gene GADD153 is upregulated in the 1-
77 methyl-4-phenyl-pyridinium (MPP$^+$) neurotoxin model [30]
78 of PD neurodegeneration. As PDI family members are also
79 upregulated by the UPR [18], we investigated the expres-
80 sion of the PDI family of proteins in toxic models of PD
81 neurodegeneration and in human brain tissue from PD and
82 DLB subjects in the current study. The PDI family of
83 proteins consists of structurally related enzymes that
84 catalyze disulfide bond formation, reduction, or isomer-
85 ization of newly synthesized proteins [10,12,34]. The PDI
86 family also acts as part of a quality-control system to ensure
87 the correct folding of proteins, a feature essential to cell
88 viability.

89 The results of this study demonstrate that PDIp is
90 uniquely upregulated among family members by either
91 MPP$^+$ or lactacystin. RT-PCR confirms PDIp expression in
92 human brain and immunohistochemical studies demon-
93 strate that PDIp immunoreactivity in LBs co-localizes with
94 α-synuclein.

95 ## 2. Materials and methods

96 ### 2.1. Materials

97 MPP$^+$ iodide, lactacystin, canavanine, Dulbecco's Modi-
98 fied Eagle's Medium (DMEM), retinoic acid (RA), Tri

99 reagent, (Sigma, St. Louis, MO), H$_2$O$_2$ (Fisher Scientific,
100 Rochester, NY), and fetal bovine serum (FBS) (Gibco BRL,
101 Grand Island, NY) were purchased from commercial sources.

102 ### 2.2. Cell culture and toxin treatment

103 The undifferentiated human neuroblastoma cell line SH-
104 SY5Y (ATCC CRL-2266) was cultured at 37 °C in a 95%
105 air, 5% CO$_2$ humidified incubator and maintained in
106 DMEM (high glucose) supplemented with 10% FBS.
107 Undifferentiated cells were routinely subcultured when
108 confluent and the culture medium was changed twice a
109 week. For toxin experiments, cells were plated into 48-well
110 plates (10,000 cells/well) and/or 100 mm^2 dishes (0.8×10^6
111 cells/dish) in differentiation media (10 ml DMEM plus 10%
112 FBS, 100 units/ml penicillin, 100 mg/ml streptomycin and
113 10 μM RA). Cells were cultured for 3 days then the media
114 were changed to fresh differentiation media. The cells were
115 cultured another 3 days and freshly prepared toxin or
116 vehicle was added in fresh differentiation media for various
117 lengths of time. All appropriate safety precautions were
118 used in handling toxin solutions.

119 ### 2.3. RNA extraction and RT-PCR

120 Total RNA was isolated from 100 mm^2 dishes in 1 ml Tri
121 Reagent (Sigma). Approximately 50 μg of RNA was treated
122 with 1 unit of DNAse-I using the MessageClean kit (Gen-
123 Hunter, Nashville, TN). DNAse-treated RNA was subjected
124 to RT-PCR analyses, as described below, using primers to
125 glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12;
126 G3PDH) to ensure that each RNA preparation was free of
127 DNA. DNAse-treated RNA (5 μg) was reverse transcribed
128 using oligo(dT) primers provided in the Superscript kit
129 (Clontech, Palo Alto, CA). Three different primer sets were
130 used for the amplification of PDIp (5′CGCCTGGTCACG-
131 GAGTTC 3′and 5′CCACAGGCGCATACTTCTTAGT 3′), (5′
132 CTTTGGACTCAAGGCTGAGG 3′ and 5′ GCTCCTGT-
133 CCAGCAGTGATG 3′), and (5′ AGCCTCCAGAGGAG-
134 GAAATC 3′ and 5′ AGGTCCTGGAAGAAGCCAAT 3′).
135 The following primers were purchased (Gibco BRL) and
136 used for the detection of the PDI family members: PDI (5′
137 AGACTCACATCCTGCTGTTCTTGCC 3′ and 5′
138 TCGGGCTTGTACTTGGTCATCTCC 3′), PDIR (5′ GAA-
139 GAGCAGCAGACAAGCCTGTTGC 3′ and 5′ AAATCC-
140 CAATTCTGTGCGGTCGCTG 3′), P5 (5′
141 CAGTCTGAACTTGAGACCGCGTTGG 3′and 5′GTCAT-
142 CAAGCTCCACATCACTGAGG 3′), ERp29 (5′
143 TCCCAAAAGCAAGTTCGTCT 3′ and 5′ GCTCCATGTT-
144 CAGCTTGTCA 3′), ERp44 (5′TCTTGATCGCAGCAAAA-
145 GAA 3′and 5′ACCATATCCGGAGCAGAATG 3′), ERp57 (5′
146 TTATTTGTGTTGGGGGAAATG 3′ and 5′ AGGGGTCT-
147 CATTTATTGTCACTG 3′), ERp72 (5′ GCATCGTTTGA-
148 CACCTTGCGGTGG 3′ and 5′
149 CAATACCAGGATGCCGCTAACAACC 3′). Conditions
150 for G3PDH amplification were identical to those previously

151 described [3]. For each primer set the reaction cycle consisted
152 of the following steps: 94 °C for 1 min, 60 °C for 1 min and 72
153 °C for 2 min. The number of PCR cycles needed for each
154 primer set was optimized to give PCR products in the linear
155 range of detection for the ChemiImaging system described
156 below. Reactions for G3PDH, PDI, P5, PDIR, ERp29, ERp72
157 were carried out for 30 cycles while reactions for PDIp,
158 ERp44, and ERp57 were carried out for 35 cycles. Following
159 resolution by electrophoresis on 2% agarose gels containing
160 0.5 µg/ml ethidium bromide, PCR products were visualized
161 and quantified using the 4400 ChemiImager low light
162 imaging system (Alpha-Innotech, San Leandro, CA). PDIp,
163 PDI, PDIR, ERp29, ERp44, ERp57, ERp72 expression was
164 expressed as a ratio to the value of G3PDH product obtained
165 from parallel reactions. RT-PCR confirmation of PDIp
166 expression in human brain used RNA isolated from frozen,
167 post-mortem, superior frontal cortex from a subject with
168 pathologically confirmed DLB (Harvard Brain Tissue
169 Resource Center). RT-PCR was also performed using cDNA
170 generated from frozen, post-mortem, whole brain lysates
171 from a control subject with non-neurological diseases
172 (Ambion, Austin, TX).

2.4. Western blots

174 Cells were harvested from 100 mm² dishes in 300 µl of
175 phosphate buffered saline (PBS) using a plastic cell lifter.
176 Harvested cells were pelleted by centrifugation ($12,000 \times g$
177 at 4 °C), resuspended in 100 µl lysis buffer (20 mM Tris pH
178 7.4, 140 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM
179 EDTA, 1× protease inhibitor cocktail (Roche, Mannheim,
180 Germany) and frozen to −70 °C. Thawed cell suspensions
181 were clarified by centrifugation ($12,000 \times g$ at 4 °C). The
182 protein concentrations of the resultant supernatants were
183 determined using the BCA kit (Pierce, Rockford, IL). Fifty
184 µg of protein were resolved on a 10–20% Tris–HCl gel and
185 transferred to nitrocellulose (Bio-Rad, Hercules, CA). After
186 transfer, gels were stained with Bio-Safe Coomassie reagent
187 (Bio Rad) to monitor equal protein loading of lysates from
188 the different experimental conditions. Membranes were
189 blocked in 5% non-fat dried milk in TBST buffer (20 mM
190 Tris, 500 mM NaCl pH 7.5 with 0.05% Tween-20) for 60
191 min at room temperature. Membranes were then washed for
192 10 min three times in TBST. Membranes were incubated
193 with 1:200 dilutions of polyclonal rabbit anti-PDIp antisera
194 [7] or polyclonal rabbit anti-PDI (Stressgen Biotechnolo-
195 gies, Victoria, BC, Canada) diluted in TBST overnight at
196 room temperature. Membranes were washed as before and
197 incubated with a 1:10,000 dilution of goat anti-rabbit
198 horseradish peroxidase (HRP) conjugated secondary anti-
199 body (Chemicon International, Temecula, CA) diluted in
200 TBST for 2 h at room temperature. Membranes were
201 washed as before and incubated with SuperSignal West Pico
202 Chemiluminescent Substrate (Pierce). Membranes were
203 visualized with the 4400 ChemiImager low light imaging
204 system (Alpha-Innotech). Exposure times were optimized to
205 collect images of protein products within the linear range of
206 detection for the Chemiimaging system.

2.5. Immunohistochemistry

208 Human brain tissue from subjects with pathologically
209 confirmed PD (five cases; ages 63–86 years; mean age, 78.6
210 years), DLB (eight cases; ages 74–87 years; mean age, 80.1
211 years), AD (six cases; ages 75–83 years; mean age, 78
212 years), and control subjects with non-neurological diseases
213 without any pathological changes associated with PD, DLB
214 or AD (four cases; ages 73–86 years; mean age, 78.2 years)
215 were obtained from the Boston University Brain Tissue
216 Resource Center and used for immunocytochemistry.
217 Sections from fresh-frozen and paraffin embedded tissues
218 were incubated with either blocking serum (same species as
219 secondary antibody) for 20 min or Super Block solution
220 (ScyTek Laboratories, UT) for 5 min. Sections were
221 incubated with either polyclonal rabbit anti-PDIp antisera
222 [7] and/or mouse monoclonal anti-α-synuclein antibody
223 (Zymed Laboratories; South San Francisco, CA) overnight
224 at 4 °C. Sections were washed in PBS plus 10% BSA prior
225 to incubation with biotinylated secondary antibodies (Vec-
226 tor, CA), Alexa Flour 488 goat anti-rabbit IgG (Molecular
227 Probes; Eugene, OR) (PDIp) or Alexa Flour 546 anti-mouse
228 IgG (Molecular Probes; Eugene, OR) at room temperature
229 for 1 h. The sections were washed as before and staining
230 was performed with an avidin–biotin complex (RTU ABC
231 elite kit, Vector, CA) and AEC system (ScyTek Laborato-
232 ries, UT) following manufacturers' instructions or detected
233 by fluorescence microscopy (Nikon Eclipse E800 with RT
234 Slider). Some sections were also counterstained with
235 hematoxylin or 4′,6-diamidino-2-phenylindole*2HCl
236 (DAPI). The specificity of anti-PDIp serum was also tested
237 by preabsorption assay (data not shown) using anti-PDIp
238 serum (1:500 in PBS plus 10% BSA) preabsorbed with 20
239 µg/ml human (6xHis)-PDIp peptide, room temperature for
240 1 h before applied as primary antibody.

2.6. Analysis of cell viability

242 Cells were plated into 48-well plates (10,000 cells/well)
243 and differentiated as described above and freshly prepared
244 toxins were added for 24 h. The conversion of soluble 3-
245 [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
246 (MTT) to insoluble formazan was assayed using the
247 CellTiter Non-Radioactive Cell Proliferation Assay (Prom-
248 ega, Madison, WI) as described previously [23].

2.7. Statistical analysis

250 Values represent the means±SEM. Differences among
251 means were analyzed by Student's *t*-test or one-way
252 ANOVA with time or treatment as the independent factor.
253 When ANOVA showed significant differences, post-hoc
254 tests were performed.

3. Results

3.1. MPP⁺ exposure and mRNA expression

Steady-state mRNA levels of each PDI family member were measured using RT-PCR with primers and RNA isolated 24, 48 and 72 h after exposure of differentiated SH-SY5Y cells to 1 mM MPP$^+$ or control conditions from their respective no toxin control. The expression of PDIp is increased at all timepoints but that of the family members PDI, PDIR, P5, ERp29, ERp44, ERp57 or ERp72 is not (Fig. 1A). Quantification of steady-state PDIp mRNA levels from four independent experiments demonstrated that the increase in PDIp in MPP$^+$-treated cultures after 24 h is $240.8 \pm 43.1\%$ (mean$\pm$SEM, one sample t-test, $p<0.05$). One-way ANOVA on the individual gene comparisons showed that PDIp was uniquely upregulated. PDIp expression did not differ significantly between 24, 48, and 72 h (Fig. 1B).

Fig. 2. Western blot analysis of PDIp and PDI expression in differentiated SH-SY5Y cells-Western blot analyses were performed using cell extracts isolated 24 h after MPP$^+$ exposure. Shown are results from a typical experiment in which lysates from control (C) and MPP$^+$-treated cells (M) were electrophoretically separated, transferred to nitrocellulose and probed with antibodies to PDIp (left panel) and PDI (right panel). Molecular weight standards in kilodaltons (kDa) are shown to the right of the blots.
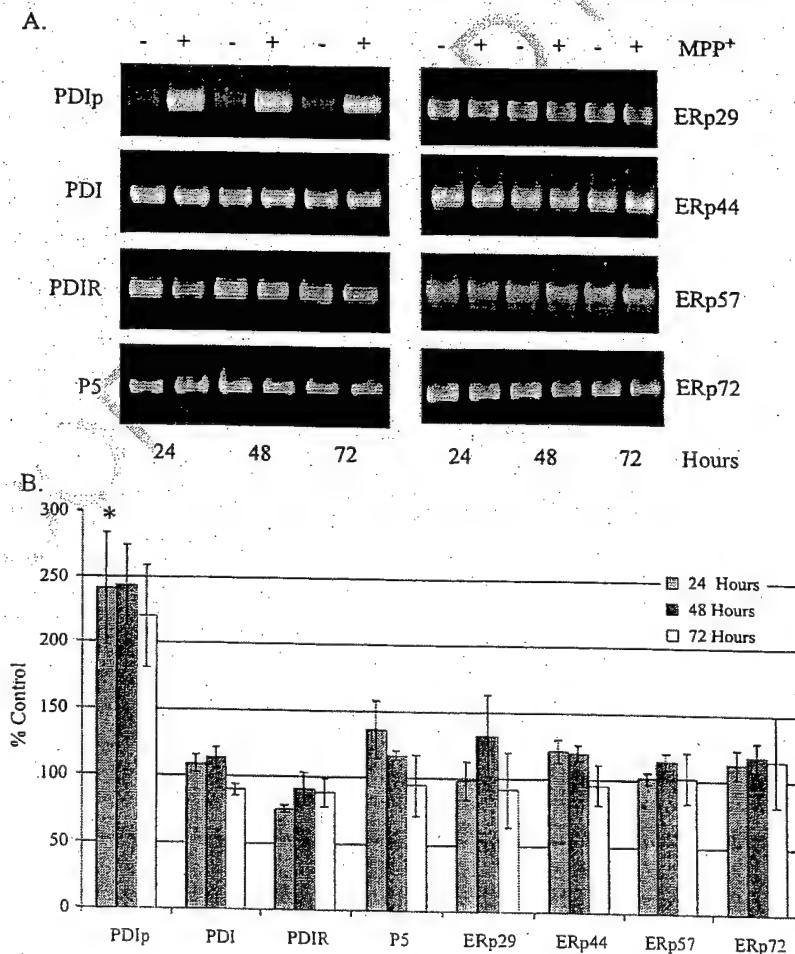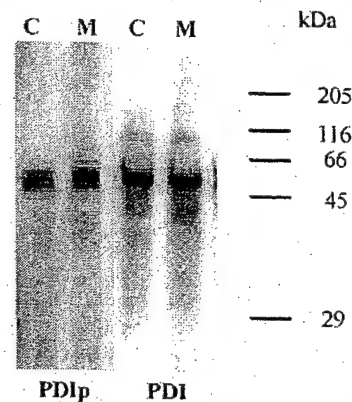
Fig. 1. RT-PCR analysis of PDIp and other PDI family member expression in differentiated SH-SY5Y cells. (A) Electrophoretic separation of PCR products from a typical RT-PCR experiment is shown using primers to PDIp, PDI, PDIR, P5, ERp29, ERp44, ERp57, ERp72 and RNA isolated 24, 48, and 72 h after exposure to 1 mM MPP$^+$ (+) or no-toxin control conditions (−). (B) Quantification of PDIp, PDI, PDIR, P5, ERp29, ERp44, ERp57, ERp72 steady-state mRNA was performed as described in the text. Data are graphed as % control$\pm$SEM where control represents those cells not treated with MPP$^+$. Values represent the mean of four independent experiments *$p<0.05$.

3.2. Western blot analysis

Western blot analysis was performed to determine the influence of MPP^+ on steady-state PDIp protein levels using protein-specific antibodies to PDIp [26] on cell lysates isolated 24 h after exposure of cells to 1 mM MPP^+ or control conditions. In parallel, steady-state PDI protein levels were also determined. Because 24 h of MPP^+ (1 mM) exposure did not alter PDI steady-state mRNA levels (Fig. 1), we did not expect steady-state PDI protein levels to change under these same experimental conditions. Both PDIp and PDI migrate with an apparent molecular weight of ~55 kDa [10]. PDIp but not PDI expression was increased in MPP^+-treated cells compared to control as shown by Western blot (Fig. 2). Coomassie staining of the SDS gels (data not shown) was done to confirm that equal amounts of protein were loaded on the gels. Quantification of steady-state PDIp and PDI protein levels from five independent experiments demonstrated that steady-state PDIp protein levels, normalized to steady-state PDI protein levels, in MPP^+-treated cultures as compared to control was increased to $129\pm8.82\%$ of control (mean$\pm$SEM, two-tailed one sample t-test $p<0.05$). Comparison of PDIp steady-state protein levels among lysates isolated 4, 12, 24 and 72 h following MPP^+ exposure (1 mM) showed that differences in steady-state PDIp protein levels as compared to control were maximal at the 24 h timepoint (data not shown).

Previous studies identified PDIp as being exclusively expressed in the pancreas [6]. To confirm that the product in our cell culture model was indeed PDIp and to determine if PDIp was also expressed in human brain tissue, RT-PCR was performed using three unique primers sets to PDIp and cDNA from three sources: (1) MPP^+-treated differentiated SH-SY5Y cells; (2) human whole-brain lysates (Ambion);
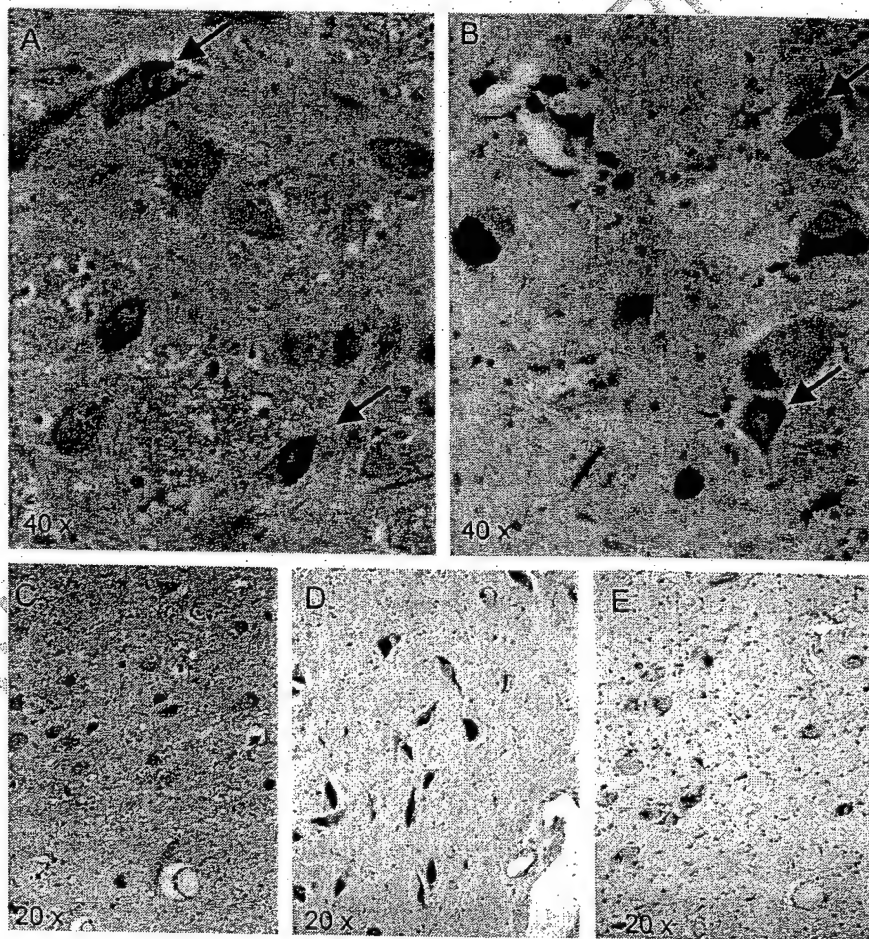

Fig. 3. PDIp immunohistochemistry in human DLB and control substantia nigra. Some 10-µm sections of DLB and control (non-diseased) brain tissues were incubated with and without antibodies to PDIp and α-synuclein. Immunoreactivity was detected using a vector ABC staining kit with AEC chromogen. The sections were also counterstained by hematoxylin. (A) Substantia nigra from DLB brain immunostained with antibodies to PDIp (40× original magnification) with typical brainstem LBs indicated by arrows. The rounded area of neuromelanin clearing indicates the position of the Lewy bodies in several neurons of the substantia nigra. Smaller dot-like inclusions in the area of the melanin clearing are immunoreactive for PDIp suggesting that PDIp stains only a portion of the LB structure. B. Substantia nigra from DLB brain immunostained with antibodies to α-synuclein (40×) with typical brainstem LBs indicated by arrows. (C) Substantia nigra from DLB brain immunostained with antibodies to PDIp (20×). (D) Substantia nigra from control (non-diseased) brain immunostained with antibodies to PDIp (20×). (E) Substantia nigra from DLB brain immunostained with secondary antibody only (20×).

305 and (3) post-mortem superior frontal cortex from a patient
306 with DLB. All three sources of cDNA produced PCR
307 products of the expected sizes with all three unique primer
308 sets (data not shown). Sequencing of the PCR products
309 produced from amplification of RNA isolated from DLB
310 brain tissue and from MPP$^+$-treated differentiated SH-SY5Y
311 cells showed 100% sequence identity to PDIp within the
312 sequences tested (bases 84–524 and 1062–1544;
313 gi5803118).

3.3. Immunohistochemical analysis

315   To determine the distribution of PDIp in human brain,
316 immunohistochemistry was performed. Positive PDIp
317 immunostaining localized to LBs was found in some nigral
318 neurons of all PD and DLB subjects examined. PDIp stains
319 discrete intraneuronal inclusions that are irregularly shaped,
320 and are not always rounded, which most likely represents
321 staining of only a portion of the Lewy body (Fig. 3A and C).
322 In addition, PDIp stains irregular threadlike neuritis in the
323 surrounding neuropil that conform to the appearance of
324 Lewy neurites. The number of PDIp positive LBs varied
325 from case to case. Positive PDIp immunostaining localized
326 to LBs was also found in the amygdala, entorhinal cortex,
327 hippocampus, and medulla of all DLB subjects examined
328 (data not shown). There was no PDIp immunostaining in the
329 SN of control subjects (Fig. 3D), or in the SN of DLB
330 subjects in sections incubated with secondary antibody only
331 (Fig. 3E). In addition, there was no PDIp immunostaining in
332 senile plaques or neurofibrillary tangles in the brains of AD
333 subjects (data not shown).
334   To estimate the subpopulation of PDIp immunoreactivity
335 aggregates in classical Lewy bodies as defined by α-
336 synuclein staining, 10 continuous, non-overlapping 20×
337 fields from individually stained, serial 10 μM sections of
338 four Parkinson's disease patients were counted. Approx-
339 imately 57% of α-synuclein immunostained LBs are also
340 immunopositive for PDIp.
341   To determine if PDIp and α-synuclein immunoreactiv-
342 ities were co-localized in LBs, a double labeling experiment
343 was performed. Sections of nigral tissue from subjects with
344 pathologically confirmed DLB were incubated with anti-
345 bodies to α-synuclein and PDIp bound to unique fluorescent
346 secondary antibodies. Positive PDIp (green) and α-synuclein
347 (red) immunostaining was identified in some LBs and the
348 overlay of the two fluorescent signals (yellow) demonstrates
349 that in those LBs where they are both present, α-synuclein
350 and PDIp immunoreactivity co-localize (Fig. 4). The
351 counterstain of nuclei with DAPI (blue) is also shown.

3.4. Lactacystin exposure and PDIp expression

353   Because LB formation in PD and DLB is associated with
354 impaired proteasome function [14.26], we tested whether
355 the highly specific proteasome inhibitor, lactacystin,
356 increased PDIp expression in differentiated SH-SY5Y cells.

Fig. 4. Co-localization of α-synuclein and PDIp expression in human DLB, substantia nigra. A double labeling experiment was performed using DLB, SN tissue immunostained with antibodies to α-synuclein (red) and PDIp (green) each bound to unique fluorescent secondary antibodies. The red staining shows α-synuclein immunoreactivity, the green staining shows PDIp staining immunoreactivity, the yellow staining shows areas where α-synuclein and PDIp immunoreactivity overlay. The counterstain of nuclei with DAPI (blue) is also shown. Typical brainstem LBs are indicated by arrows (40× magnification).

357 Cells were treated with 10 μM lactacystin or no-toxin
358 control conditions for 24 h and RNA was isolated for RT-
359 PCR analysis (Fig. 5). Quantification of steady-state PDIp
360 mRNA levels from three independent experiments demon-
361 strate that lactacystin increases PDIp expression by
362 148.7±1.9% compared to the control (mean±SEM, one
363 sample t-test, $p<0.002$, $n=3$). PDIp expression did not
364 increase in differentiated SH-SY5Y cells following expo-
365 sure for 24 h to the oxidant H_2O_2 (600 μM) or to the inducer
366 of misfolded proteins canavanine (10 mM) even though
367 both agents display similar toxicity with lactacystin (data
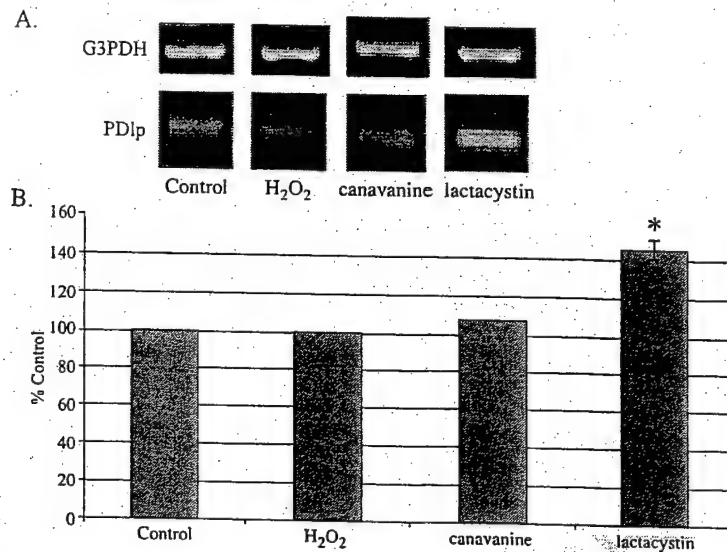368 not shown).

Fig. 5. Expression of PDIp in differentiated SH-SY5Y cells following exposure to H_2O_2, canavanine, and lactacystin. (A) Electrophoretic separation of PCR products from a typical RT-PCR experiment is shown using primers to G3PDH and PDIp and RNA isolated 24 h after exposure to control conditions (no toxin), H_2O_2 (600 μM), canavanine (10 mM) and lactacystin (10 μM). (B) Quantification of PDIp steady-state mRNA was performed as described in the text. Data are graphed as % control±SEM where control represents those cells not treated with MPP^+. Values for lactacystin represent the mean of three independent experiments *$p<0.002$. Values for H_2O_2 and canavanine represent the mean of two independent experiments.

369 Western blot analysis was performed to determine the
370 influence of lactacystin on steady-state PDIp and PDI
371 protein levels using cell lysates isolated 24 h after exposure
372 of cells to 10 μM lactacystin or control conditions. PDIp but
373 not PDI steady-state protein levels increased following
374 lactacystin exposure (data not shown). Quantification of
375 steady-state PDIp and PDI protein levels from four
376 independent experiments demonstrated that steady-state
377 PDIp protein levels, normalized to steady-state PDI protein
378 levels, in lactacystin-treated cultures as compared to control
379 was increased to 130.3±3.12% of control (mean±SEM,
380 two-tailed one sample t-test $p<0.05$).

381 ### 4. Discussion

382 This study shows that the PDI family member PDIp is
383 uniquely upregulated among family members within 24 h
384 following exposure of differentiated SH-SY5Y cells to
385 either MPP^+ or lactacystin. In contrast, neither H_2O_2 nor
386 canavanine increased PDIp expression despite decreasing
387 cell viability to a comparable degree. Together, this
388 suggests that PDIp upregulation may be a specific signal
389 for proteasomal impairment. This hypothesis is supported
390 by our recent unpublished observation that exposure of SH-
391 SY5Y cells to the proteasome inhibitor, MG115 [23] also
392 increases PDIp steady-state mRNA (~140% control after 24
393 h exposure to 30 μM). Cell death following canavanine, we
394 speculate, is likely due to the generation of several
395 defective polypeptides (not just those associated with the
396 proteasome) because canavanine (the beta-oxa-analog of L-
397 arginine and a substrate for arginyl tRNA synthetase) is

incorporated into all nascent proteins in place of L-arginine 420
[1]. 421
Western blot analysis of total cell lysates confirmed that 422
PDIp steady-state protein levels are up-regulated in response 423
to both MPP^+ and lactacystin; though the magnitude of 424
steady-state protein upregulation was less than that of 425
steady-state mRNA. The difference between the magnitude 426
of induction between steady-state protein and mRNA levels 427
has also been found with other ER stress genes [3] and may 428
be related to the observation that induction of ER stress 429
genes is accompanied by phosphorylation of the eukaryotic 430
initiation factor-2α (eIF-2α), which results in an overall 431
decrease in protein translation [18]. It remains to be seen 432
whether the ~30% increase in steady-state PDIp protein 433
levels following toxin exposure results in a proportional 434
increase in PDIp activity. 435
It is unclear why MPP^+ and lactacystin increase PDIp 436
expression but not the expression of other PDI family 437
members. One possibility is that the different PDI related 438
proteins fulfill similar functions but act on specific substrates. 439
PDIp is structurally and functionally related to PDI. PDI and 440
PDIp share the same domain organization (45% sequence 441
identity) but PDIp lacks an enriched acidic amino acid stretch 442
at the carboxyl terminus that functions as a peptide-binding 443
domain [28]. In addition, the PDIp cDNA contains three 444
potential N-linked glycosylation sites not found in typical 445
mammalian PDIs. Both PDI and PDIp have been shown to 446
specifically interact with radiolabeled model peptides and 447
misfolded proteins in vitro; however, PDIp but not PDI has 448
high affinity for peptides with hydroxyaryl groups [19]. 449
Characterizing protein–protein interactions will provide 450
insight into the possible protein substrates of brain PDIp. 451

452 Alternatively, the members of the PDI family might be
453 differentially regulated. For example, some PDI-related
454 proteins are upregulated by the UPR [22,24]. Future
455 identification of the responsive promoter element(s) of a
456 PDIp gene may provide further insights into the regulatory
457 molecules that signal the initial events in dopaminergic
458 degeneration. In addition, if upregulation of PDIp is found
459 to be exclusively located with in the dopaminergic cells in
460 the SN, characterization of the PDIp promoter elements may
461 provide the basis for expressing protective genes in
462 dopaminergic cells affected in PD.

463 In addition to being expressed in SH-SY5Y cells, PDIp
464 steady-state mRNA was detected in human brain by RT-
465 PCR. The sensitivity of RT-PCR to detect low levels of
466 mRNA transcripts may account, in part, for our discovery
467 that PDIp is expressed in the brain. This result contrasts with
468 Northern blot experiments that suggested PDIp expression
469 was restricted to pancreatic tissue [6]. We have used two
470 methods to help clarify whether PDIp immunoreactivity in
471 the brain represents neuronal expression. First, we have
472 used laser capture microdisection to isolate RNA from Lewy
473 body containing neurons of the substantia nigra and used
474 this RNA to detect PDIp expression by RT-PCR (unpub-
475 lished results). Secondly, we have cultured mouse cortical
476 neurons and have shown that MPP$^+$ increases the expression
477 of murine PDIp in these primary neuronal cultures
478 (unpublished results).

479 Positive PDIp immunostaining localized to LBs was
480 found in some nigral neurons of all PD and DLB subjects
481 examined. This restricted distribution of PDIp immunor-
482 eactivity may be the result of increased PDIp expression in
483 response to ongoing exposure to toxic factor(s) in those
484 neurons and/or the sequestration of PDIp to the LB over
485 time. PDIp immunoreactivity is detected in both brainstem
486 and cortical LBs and co-localizes with α-synuclein immu-
487 noreactivity. The co-localizaiton of α-synuclein and PDIp
488 suggests that these molecules may have close physical
489 interactions. Although it is not clear why only some LBs are
490 immunopositive for PDIp, it may represent the existence of
491 different stages of LBs development. Sequestration of PDIp
492 within the LB could lead to a loss of PDIp function.

493 The physiologic role of PDIp in the brain is not known.
494 PDIp is highly expressed in the acinar cells of the pancreas
495 [7]. PDIp expression in both pancreatic acinar cells and in
496 dopaminergic neurons may reflect the largely secretory
497 function of both types of cells. Pancreatic acinar cells secrete
498 digestive enzyme zymogens, and dopaminergic neurons
499 secrete DA in response to hormonal and neural signals
500 (regulated secretion); which differs from the continuous
501 movement of proteins to the cell surface in transport vesicles
502 (constitutive secretion) [2]. This suggests the possibility that
503 in DA neurons PDI may facilitate the folding of constitu-
504 tively secreted proteins and PDIp may facilitate the folding
505 of regulated secreted proteins in the ER.

506 The connection between PDIp expression and protea-
507 some dysfunction is also unknown. One possibility is that

508 PDIp functions as chaperone in the unfolding of protein
509 substrates, such as tyrosine hydroxylase, destined for
510 degradation by the proteasome [8]. The 26S proteasome is
511 relatively ineffective at degrading aggregated proteins [20]
512 and requires that substrates be unfolded to gain access to the
513 active site and. This would require that PDIp have access to
514 the cytoplasm. The subcellular distribution of PDIp in DA
515 neurons remains to be determined; however, at least two
516 other members of the PDI family, PDI and ERp57 are found
517 in the cytosol [34]. It is also possible that PDIp exists in the
518 ER as part of a complex containing both ER membrane and
519 cytosolic components involved in degrading misfolded ER
520 proteins. Data indicating that PDI is involved in such a
521 complex have recently been published [27,34].

522 In summary, we show that the PDI family member PDIp
523 is uniquely upregulated among family members within 24
524 h following exposure of differentiated SH-SY5Y cells to 1
525 mM MPP$^+$. Comparisons of PDIp expression among
526 cultures treated with lactacystin suggest that impaired
527 proteasome function may be important mechanistically in
528 the up-regulation of PDIp. Gene expression and immuno-
529 histochemical studies using postmortem brain tissue
530 demonstrate that PDIp is expressed in the brain and that
531 PDIp immunoreactivity is associated with LB pathology.
532 Collectively, these findings suggest that increased PDIp
533 expression in proteasome impaired DA neurons may
534 participate in LB formation and perhaps contribute to
535 neurodegeneration. The identification of the structural
536 components of the inclusions found in the brains of
537 individuals afflicted with neurodegenerative diseases pro-
538 vides insight into the pathological mechanism involved.
539 The identification of the PDI family member, PDIp, may
540 provide a new understanding of the pathological mecha-
541 nism involved in LB formation.

References 548
549

[1] A.K. Bence, P.A. Crooks, The mechanism of L-canavanine cytotox- 550
icity: arginyl tRNA synthetase as a novel target for anticancer drug 551
discovery, J. Enzyme Inhib. Med. Chem. 18 (2003) 383–394. 552
[2] T.L. Burgess, R.B. Kelly, Constitutive and regulated secretion of 553
proteins, Annu. Rev. Cell Biol. 3 (1987) 243–293. 554
[3] K.J. Conn, W.W. Gao, M.D. Ullman, C. McKeon-O'Malley, P.B. 555
Eisenhauer, R.E. Fine, J.M. Wells, Specific upregulation of 556
GADD153/CHOP in MPP$^-$-treated SH-SY5Y cells, J. Neurosci. 557
Res. 68 (2001) 755–760. 558
[4] W. Dauer, S. Przedborski, Parkinson's disease: mechanisms and 559
models, Neuron 39 (2003) 889–909. 560

561 [5] T.M. Dawson, V.L. Dawson, Molecular pathways of neurodegenera-
562   tion in Parkinson's disease, Science 302 (2003) 819–822.
563 [6] M.G. Desilva, J. Lu, G. Donadel, W.S. Modi, H. Xie, A.L. Notkins,
564   M.S. Lan, Characterization and chromosomal localization of a new
565   protein disulfide isomerase, PDIp, highly expressed in human
566   pancreas, DNA Cell Biol. 15 (1996) 9–16.
567 [7] M.G. Desilva, A.L. Notkins, M.S. Lan, Molecular characterization of
568   a pancreas-specific protein disulfide isomerase, PDIp, DNA Cell Biol.
569   16 (1997) 269–274.
570 [8] A.P. Doskeland, T. Flatmark, Ubiquitination of soluble and mem-
571   brane-bound tyrosine hydroxylase and degradation of the soluble
572   form, Eur. J. Biochem. 269 (2002) 1561–1569.
573 [9] G. Fenteany, S.L. Schreiber, Lactacystin, proteasome function, and
574   cell fate, J. Biol. Chem. 273 (1998) 8545–8548.
575 [10] D.M. Ferrari, H.D. Soling, The protein disulphide-isomerase family:
576   unravelling a string of folds, Biochem. J. 339 (Pt 1) (1999) 1–10.
577 [11] L.S. Forno, Neuropathology of Parkinson's disease, J. Neuropathol.
578   Exp. Neurol. 55 (1996) 259–272.
579 [12] R.B. Freedman, T.R. Hirst, M.F. Tuite, Protein disulphide isomerase:
580   building bridges in protein folding, Trends Biochem. Sci. 19 (1994)
581   331–336.
582 [13] W.R. Gibb, A.J. Lees, The relevance of the Lewy body to the
583   pathogenesis of idiopathic Parkinson's disease, J. Neurol. Neurosurg.
584   Psychiatry 51 (1988) 745–752.
585 [14] B. Halliwell, Hypothesis: proteasomal dysfunction: a primary event in
586   neurogeneration that leads to nitrative and oxidative stress and
587   subsequent cell death, Ann. N.Y. Acad. Sci. 962 (2002) 182–194.
588 [15] A. Hershko, A. Ciechanover, The ubiquitin system, Annu. Rev.
589   Biochem. 67 (1998) 425–479.
590 [16] M. Hochstrasser, Ubiquitin-dependent protein degradation, Annu.
591   Rev. Genet. 30 (1996) 405–439.
592 [17] W.A. Holtz, K.L. O'Malley, Parkinsonian mimetics induce aspects of
593   unfolded protein response in death of dopaminergic neurons, J. Biol.
594   Chem. 278 (2003) 19367–19377.
595 [18] R.J. Kaufman, Stress signaling from the lumen of the endoplasmic
596   reticulum: coordination of gene transcriptional and translational
597   controls, Genes Dev. 13 (1999) 1211–1233.
598 [19] P. Klappa, R.B. Freedman, M. Langenbuch, M.S. Lan, G.K.
599   Robinson, L.W. Ruddock, The pancreas-specific protein disulphide-
600   isomerase PDIp interacts with a hydroxyaryl group in ligands,
601   Biochem. J. 354 (2001) 553–559.
602 [20] R.R. Kopito, R. Sitia, Aggresomes and Russell bodies, Symptoms of
603   cellular indigestion? EMBO Rep. 1 (2000) 225–231.
604 [21] A.E. Lang, A.M. Lozano, Parkinson's disease. Second of two parts,
605   N. Engl. J. Med. 339 (1998) 1130–1143.
606 [22] N. Lenny, M.Green, Regulation of endoplasmic reticulum stress
607   proteins in COS cells transfected with immunoglobulin mu heavy
608   chain cDNA, J. Biol. Chem. 266 (1991) 20532–20537.
609 [23] U.G. Lopes, P. Erhardt, R. Yao, G.M. Cooper, p53-dependent
610   induction of apoptosis by proteasome inhibitors, J. Biol. Chem. 272
611   (1997) 12893–12896.
660

612 [24] R.A. Mazzarella, N. Marcus, S.M. Haugejorden, J.M. Balcarek, J.J.
613   Baldassare, B. Roy, L.J. Li, A.S. Lee, M. Green, Erp61 is GRP58, a
614   stress-inducible luminal endoplasmic reticulum protein, but is devoid
615   of phosphatidylinositide-specific phospholipase C activity, Arch.
616   Biochem. Biophys. 308 (1994) 454–460.
617 [25] K.S. McNaught, C.W. Olanow, B. Halliwell, O. Isacson, P. Jenner,
618   Failure of the ubiquitin-proteasome system in Parkinson's disease,
619   Nat. Rev., Neurosci. 2 (2001) 589–594.
620 [26] K.S. McNaught, C. Mytilineou, R. Jnobaptiste, J. Yabut, P.
621   Shashidharan, P. Jennert, C.W. Olanow, Impairment of the ubiqui-
622   tin-proteasome system causes dopaminergic cell death and inclusion
623   body formation in ventral mesencephalic cultures, J. Neurochem. 81
624   (2002) 301–306.
625 [27] M. Molinari, C. Galli, V. Piccaluga, M. Pieren, P. Paganetti,
626   Sequential assistance of molecular chaperones and transient formation
627   of covalent complexes during protein degradation from the ER, J. Cell
628   Biol. 158 (2002) 247–257.
629 [28] R. Noiva, R.B. Freedman, W.J. Lennarz, Peptide binding to protein
630   disulfide isomerase occurs at a site distinct from the active sites,
631   J. Biol. Chem. 268 (1993) 19210–19217.
632 [29] M.S. Pollanen, D.W. Dickson, C. Bergeron, Pathology and biology of
633   the Lewy body, J. Neuropathol. Exp. Neurol. 52 (1993) 183–191.
634 [30] S. Przedborski, M. Vila, The 1-methyl-4-phenyl-1,2,3,6-tetrahydro-
635   pyridine mouse model: a tool to explore the pathogenesis of
636   Parkinson's disease, Ann. N.Y. Acad. Sci. 991 (2003) 189–198.
637 [31] E.J. Ryu, H.P. Harding, J.M. Angelastro, O.V. Vitolo, D. Ron, L.A.
638   Greene, Endoplasmic reticulum stress and the unfolded protein
639   response in cellular models of Parkinson's disease, J. Neurosci. 22
640   (2002) 10690–10698.
641 [32] M.Y. Sherman, A.L. Goldberg, Cellular defenses against unfolded
642   proteins: a cell biologist thinks about neurodegenerative diseases,
643   Neuron 29 (2001) 15–32.
644 [33] M.G. Spillantini, R.A. Crowther, R. Jakes, M. Hasegawa, M. Goedert,
645   alpha-Synuclein in filamentous inclusions of Lewy bodies from
646   Parkinson's disease and dementia with lewy bodies, Proc. Natl. Acad.
647   Sci. U. S. A. 95 (1998) 6469–6473.
648 [34] C. Turano, S. Coppari, F. Altieri, A. Ferraro, Proteins of the PDI
649   family: unpredicted non-ER locations and functions, J. Cell. Physiol.
650   193 (2002) 154–163.
651 [35] E.M. Valente, P.M. bou-Sleiman, V. Caputo, M.M. Muqit, K. Harvey,
652   S. Gispert, Z. Ali, T.D. Del, A.R. Bentivoglio, D.G. Healy, A.
653   Albanese, R. Nussbaum, R. Gonzalez-Maldonado, T. Deller, S. Salvi,
654   P. Cortelli, W.P. Gilks, D.S. Latchman, R.J. Harvey, B. Dallapiccola,
655   G. Auburger, N.W. Wood, Hereditary early-onset Parkinson's disease
656   caused by mutations in PINK1, Science 304 (2004) 1158–1160.
657 [36] D. Voges, P. Zwickl, W. Baumeister, The 26S proteasome: a molecular
658   machine designed for controlled proteolysis, Annu. Rev. Biochem. 68
659   (1999) 1015–1068.

Upregulation of Clusterin/apolipoprotein J in Lactacystin-treated SH-SY5Y Cells

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RUNNING TITLE: Lactacystin induces clusterin expression

ABSTRACT:

Clusterin (apolipoprotein J) is a highly conserved, multifunctional, vertebrate glycoprotein. Several isoforms of clusterin have been described including the predominant secreted isoform (sCLU) and several nuclear isoforms (nCLU) associated with cell death. sCLU has been shown to bind a variety of partly unfolded, stressed proteins including those associated with Lewy bodies (LBs) in patients with Parkinson's disease (PD). The development of familial and sporadic PD has been associated with the ubiquitin-proteasome system (UPS) dysfunction and aberrant protein degradation. This suggests that failure of the UPS to degrade abnormal proteins may underlie nigral degeneration and LB formation in PD. To explore this concept, the effects of toxin-mediated proteasomal impairment on changes in gene expression and cell viability were studied in differentiated SH-SY5Y cells. Clusterin expression was increased in cells exposed for 24 hours to the proteasomal inhibitor lactacystin (10 μM) as determined by gene microarray analysis. RT-PCR showed that sCLU, not nCLU, was the major clusterin isoform expressed in both control and lactacystin treated cells. Western blot analysis identified statistically significant increases in sCLU in total cell lysates after 24 hours of lactacystin exposure and showed that sCLU fractionates with the endoplasmic reticulum. Time course studies demonstrated that maximal decreases (4 hours) in proteasome activity preceded maximal increases (24 hours) in clusterin expression. Together these data suggest that proteasome impairment contributes significantly to the upregulation of sCLU in SH-SY5Y cells supporting the hypothesis that the association of clusterin with LBs in PD may be related to UPS failure.

KEY WORDS: Parkinson's disease, Proteasome, Gene expression

INTRODUCTION:

Clusterin is a highly conserved, nearly ubiquitous, vertebrate protein known by numerous names including apolipoprotein J (ApoJ) (Jones and Jomary, 2002). The expression of the clusterin gene (CLU) is complex, resulting in different clusterin isoforms that appear in different cell compartments. Translated as a 449 amino acid precursor, clusterin possesses a signal peptide that guides it into the endoplasmic reticulum (ER) and is glycosylated and internally cleaved before it is secreted. The secreted form of the clusterin protein (sCLU) is a heterodimeric sulfated 80 kDa glycoprotein that appears as a ~40 kDa α- and β-protein smear by SDS-PAGE under reducing conditions (Yang et al., 1999). sCLU possesses nonspecific binding activity to hydrophobic domains of various proteins *in vitro* (Humphreys et al., 1999) and has been hypothesized to function as a molecular chaperone (Bailey et al., 2001; Clark and Griswold, 1997; Kimura et al., 1997; Michel et al., 1997).

In addition to sCLU, several intracellular clusterin isoforms have been described (Debure et al., 2003; Leskov et al., 2003; Reddy et al., 1996; Senut et al., 1992). These include several nuclear isoforms associated with cell death. For example, a 42-kDa nuclear glyco/isoform of clusterin is up-regulated in epithelial cells undergoing apoptosis (Lakins et al., 1998). Similarly, a 45 kDa isoform of clusterin has been described which, when targeted to the nucleus, can decrease cell proliferation and promote cell detachment-induced apoptosis in human prostate epithelial cells (Caccamo et al., 2003; Caccamo et al., 2004). Lastly, it has been shown that a spliced variant of sCLU, lacking exon II, gives rise to a nuclear form of clusterin (nCLU) that is targeted to the nucleus

following ionizing radiation (Leskov et al., 2003) and that overexpression of nCLU results in caspase-3-independent apoptosis (Yang et al., 2000).

Besides being associated with cell death, clusterin has been related to numerous other biological processes including cytoprotection, sperm maturation, lipid transport, regulation of the complement cascade, membrane recycling, immune regulation, cell adhesion, and morphological transformation (Rosenberg and Silkensen, 1995). Clusterin gene transcription is responsive to cellular stress (Michel et al., 1997) and is induced after tissue injury and in many disease states such as glomerulonephritis, polycystic kidney disease, renal tubular injury, atherosclerosis, myocardial infarction and neurodegenerative conditions including Alzheimer's disease (AD) where it associated with amyloid plaques (Choi-Miura et al., 1992; Lidstrom et al., 1998; May and Finch, 1992; McGeer et al., 1992; Oda et al., 1994; Rosenberg and Silkensen, 1995). In addition, clusterin has been found in glial cytoplasmic inclusions (GCIs) in cases with multiple system atrophy (MSA) and is associated with Lewy bodies in patients with dementia with Lewy bodies (DLB) and Parkinson's disease (PD) (Sasaki et al., 2002).

The pathological hallmarks of PD are the degeneration of dopamine (DA)-containing neurons in the substantia nigra (SN) and the presence of Lewy bodies (LBs) in many of the surviving DA neurons (Forno, 1996). The majority of PD cases are sporadic. The identification of a number of genes associated with rare familial forms of PD, however, has provided important insights into the underlying mechanisms of the disease. These genes, encoding α-synuclein, parkin, UCH-L1, DJ-1, and PINK1 have implicated protein misfolding, oxidative stress, and impairment of the ubiquitin-proteasome system (UPS), in the pathogenesis of the disease (Dauer and Przedborski, 2003; Dawson and

Dawson, 2003; Halliwell, 2002; McNaught et al., 2001; Valente et al., 2004). The ubiquitin-proteasome system (UPS) is the primary biochemical pathway responsible for the degradation of normal and abnormal intracellular proteins (Hershko and Ciechanover, 1998; Hochstrasser, 1996). Proteolysis by the proteasome is specifically inhibited by the *Streptomyces* metabolite lactacystin (Fenteany and Schreiber, 1998). UPS failure leads to protein accumulation, cell death (Sherman and Goldberg, 2001; Voges et al., 1999) and possibly LB formation (Alves-Rodrigues et al., 1998; Andersen, 2000; Carrell and Lomas, 1997; Dickson, 2001).

MATERIALS AND METHODS:

Materials- Lactacystin, Dulbecco's modified Eagle's Medium (DMEM), retinoic acid (RA), Tri-reagent (Sigma-Aldrich; St. Louis, MO), and fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) were purchased from commercial sources.

Cell culture, differentiation, and toxin treatment - The undifferentiated human neuroblastoma cell line SH-SY5Y (ATCC CRL-2266) was cultured and differentiated with RA as described previously (Conn et al., 2003). Briefly, undifferentiated cells were maintained in DMEM-high glucose supplemented with 10% FBS. For toxin experiments 0.8 x 10^6 cells were plated into 100 mm^2 dishes (Corning; Cambridge, MA) in differentiation media (10 ml DMEM plus 10% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin and 10 μM RA) and cultured for 6 days, with one media change, prior to

the addition of freshly prepared lactacystin (10 μM) or vehicle, phosphate buffered saline (PBS), for various lengths of time in fresh differentiation media.

Microarray analysis- 15 x 10^6 cells were treated for 24 hours with lactacystin (10 μM) or control conditions (vehicle) prior to being harvested for custom gene microarray analysis using the plastic human 12K microarray (BD Biosciences; Mt. View, CA).

RNA isolation and reverse transcription – RNA isolation and reverse transcription was performed as previously described (Conn et al., 2001). Briefly, total RNA was isolated from each experimental condition using Tri reagent (Sigma-Aldrich; St. Louis, MO) and treated with 1 unit DNAse-I using the MessageClean kit (GenHunter Corp; Nashville, TN). DNAse-treated RNA was subjected to PCR analyses, as described below, using primers to G3PDH to ensure that each RNA preparation was free of DNA. DNAse-treated RNA (5 μg) was reverse transcribed using oligo(dT) primers provided in the Superscript kit (Clonetech; Palo Alto, CA).

PCR amplification, visualization and quantification – PCR reactions for the amplification of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; G3PDH) were performed as described previously (Conn et al., 2001). Clusterin expression was detected using a primer set previously shown to distinguish sCLU from nCLU (Leskov et al., 2003) and with primers designed (Rozen and Skaletsky, 2000) to clusterin (gi20455818) corresponding to base pairs (bp) 2417 to 2436 (5' TCACTGAGGTGGTCGTGAAG 3') and 2661 and 2642 (5' TACTTGGTGACGTGCAGAGC 3'). PCR conditions were

optimized to give products in the linear range of detection using the 4400 ChemiImager low-light imaging system (Alpha-Innotech, San Leandro, CA): 30 cycles of 95 °C for 30 seconds, 68 °C for 45 seconds, and 72 °C for 45 seconds using the primers described by Leskov *et al* and 30 cycles of 94 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 2 minutes using the primers described above.

Subcellular fractionation- Enriched nuclear, endoplasmic reticular, and soluble cytosolic fractions were isolated as described previously (Rao et al., 2001). Briefly, cells were harvested from 100 mm^2 dishes in ice-cold fractionation buffer, pH 7.4 (20 mM HEPES, 10 mM KCl, 250 mM sucrose, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 X protease inhibitor cocktail) and homogenized on ice with 25 passes of a 2-ml Kontes Dounce Tissue Grinder (Fisher Scientific; Rochester, NY). After cell lysis, nuclei were pelleted by centrifugation for 10 minutes (750 × g at 4 °C) and resuspended in nuclear lysis buffer, pH 7.5 (20 mM Tris, 420 mM KCl, 1.5 mM MgCl2, 20% glycerol, 0.5% triton X100, and 1 X protease inhibitor cocktail). The supernatant was collected from the nuclear pellet and clarified by centrifugation for 30 minutes (10,000 × g at 4 °C). The resultant supernatant was collected and centrifuged for 60 minutes (100,000 × g at 4 °C) to obtain an ER pellet and a soluble cytoplasmic supernatant. The ER pellet was lysed in ER buffer, pH 7.4 (10 mM Tris 0.5% triton X100 and 1 X protease inhibitor cocktail). The protein concentrations of the enriched fractions were determined using the BCA kit (Pierce; Rockford, IL) and equal amounts of protein from each fraction were subject to Western blot analysis as described below.

Western blot analysis- Western blot analysis was performed as described previously (Conn et al., 2001). Briefly, cells were harvested in lysis buffer (20 mM Tris pH 7.4, 140 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 X protease inhibitor cocktail) and 50 µg of protein from each experimental condition were resolved by SDS PAGE and electrophoretically transferred to nitrocellulose membranes. After blocking membranes with 5% non-fat milk, membranes were incubated with either a 1µg/ml dilution of polyclonal goat anti-apolipoprotein J (Chemicion International; Temecula, CA) or a 1:1,000 dilution of polyclonal rabbit anti-PDI (Stressgen Biotechnologies; Victoria, BC Canada). After washing, membranes were incubated with 1:10,000 dilution of donkey anti-goat or goat anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (Chemicon International Inc; Temecula, CA), respectively. Membranes were washed prior to incubation with SuperSignal West Pico Chemiluminescent Substrate (Pierce; Rockland, IL). Membranes were visualized with the 4400 ChemiImager low light imaging system (Alpha-Innotech; San Leandro, CA). Exposure times were optimized to collect images of protein products within the linear range of detection for the Chemiimaging system.

20S Proteasome Activity Assay- Cells were harvested from 100 mm^2 dishes in 300 µl of PBS using a plastic cell lifter. Harvested cells were pelleted by centrifugation (12,000 x g at 4 °C), resuspended in 100 µl lysis buffer (PBS with 1% Triton X-100) and frozen to -70 °C. Thawed cell suspensions were sonicated using a Branson sonifier 450 (30 seconds with a 50% duty cycle at a power level of 20 W) and clarified by centrifugation (12,000 x g at 4 °C). The protein concentrations of the resultant supernatants was determined using

the BCA kit (Pierce; Rockford, IL). The supernatant was assayed for proteasome activity using the 20S Proteasome Activity Assay Kit (Chemicon; Temecula, CA) according to the manufacturer's instructions. Briefly, 20 µg of protein from control (con) and each experimental (exp) condition was incubated with (+) and without (-) 3.125 µM lactacystin (lac) for 10 minutes at room temerature prior to being incubated with 5 µM of the LLVY-7-amino-4-methylcoumarin (AMC) flourogenic substrate at 60 °C for 60 minutes. Flourescence was quantified as relative fluorescence units (RFU) using a 355/460 nm filter set in a microtiter plate flourometer MFX (Dynex Technologies; Chentilly, VA) Proteasome activity, expressed as % control, was caluculated as: $(RFU_{exp-lac} - RFU_{exp+lac} / RFU_{con-lac} - RFU_{con+lac}) \times 100$.

Cell viability assay- The MTT assay was used to evaluate cell viability. Cells were plated at a density of 1×10^4 cells/well in 48-well tissue culture plates in 500 µl of differentiation media. Cells were cultured for various lengths of time and the conversion of soluble 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to insoluble formazan by mitochondrial dehydrogenase activity was assayed using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega; Madison, WI).

Statistical Analysis- Values represent the means ± SEM. Differences among means were analyzed by student's t-test or one-way ANOVA with time or treatment as the independent factor. When ANOVA showed significant differences, post-hoc tests were performed.

RESULTS:

To explore the hypothesis that UPS failure may underlie nigral degeneration and LB formation in PD, we studied the effects of toxin-mediated proteasomal impairment on changes in gene expression and cell viability. RA-differentiated SH-SY5Y cells were treated for 24 hours with lactacystin (10 μM) or control conditions (vehicle) prior to being harvested for custom gene microarray analysis using the plastic human 12K microarray (BD Biosciences; Mt. View, CA). Of the 683 genes identified by the manufacturer as differentially expressed, the clusterin gene showed the greatest change (Figure 1).

To confirm the gene microarray results RT-PCR analyses were performed using RNA isolated from cells treated with and without 10 μM lactacystin for 0.5, 2, 4, 8, 16, and 24 hours and primers corresponding to bp 2417 to 2436 and 2661 and 2642 of the clusterin gene (gi20455818). Parallel PCR reactions were also performed using primers to the housekeeping gene G3PDH to determine the specificity of the gene expression response. The results from a typical RT-PCR experiment using primers to clusterin and G3PDH (Figure 2A) show that lactacystin does not alter the expression of G3PDH. In contrast, clusterin gene expression was increased in lactacystin-treated cells as compared to control cells at 8, 16 and 24 hours.

To study what isoform(s) of clusterin were expressed in control and lactacystin-treated cells, RT-PCR analysis was performed using RNA isolated from cells treated with and without 10 μM lactacystin for 24 hours and primers to clusterin previously shown to amplify both the full length secreted form of clusterin, sCLU, and the alternatively

spliced, cell-death, nuclear isoform of clusterin, nCLU (Leskov et al., 2003). The results from a typical RT-PCR experiment (Figure 2B) show that a single PCR product was detected using RNA from both control and lactacystin-treated cells corresponding to the expected size of the sCLU isoform.

To determine apparent molecular mass of the clusterin isoform(s) expressed in control and lactacystin treated SH-SY5Y cells and to determine the influence of lactacystin on steady-state clusterin protein levels, Western blot analyses were performed using total cell lysates isolated 24 hours after exposure of cells to lactacystin (10 µM) or control conditions and polyclonal antibodies to recombinant sCLU. The results from a typical Western blot experiment (Figure 2C) show that the major clusterin polypeptide in both control and lactacystin treated cells migrates with an apparent molecular mass of ~40 to 45 kDa and that steady-state clusterin protein levels increase in SH-SY5Y cells following lactacystin exposure. Quantification of steady-state clusterin protein levels from four independent experiments demonstrated that the increase in clusterin in lactacystin-treated cultures after 24 hours is 124.7% ± 6.67% (mean ± SEM, one sample t-test, $p < 0.05$).

The subcellular localization of clusterin in cells exposed for 24 hours to control or lactacystin (10 µM) was examined by Western blot using enriched nuclear, ER, and soluble cytosolic fractions and antibodies to clusterin. The result from a typical Western blot experiment (Figure 2D) show that the major clusterin polypeptide in both control and lactacystin treated cells fractionates with the ER. No clusterin was found in the nuclear or soluble cytosol fractions. The integrity of the fractions was monitored in parallel Western

blot experiments using antibodies to the ER resident protein disulfide isomerase and by Coomassie staining of gels after transfer (data not shown).

The temporal relationship between clusterin gene expression, 20S proteasome activity, and cell viability was evaluated in RA-differentiated SH-SY5Y cells treated with and without 10 µM lactacystin for 0.5, 2, 4, 8, 16, 24, and 48 hours (Figure 3). Maximal decreases in proteasome activity were detected 4 hours after lactacystin exposure (38.7 % ± 3.92%; mean ± SEM, two-tailed one sample t-test p<0.0001) and preceded maximal increases in clustering gene expression at 24 hours (193.1% ± 18.2%; mean ± SEM, one sample t-test, p<0.005). Maximal changes in clusterin gene expression and proteasomal activity both preceded decreases in cell viability as measured by the MTT which is not detected until 24 hours after lactacystin exposure (76.3% ± 3.38%; mean ± SEM, two-tailed one sample t-test p<0.0005).

DISCUSSION:

The results of this study indicate that proteasome impairment contributes significantly to the upregulation of clusterin by lactacystin. This is of particular interest in furthering our understanding of the pathogenesis of PD as it suggests that the association of clusterin with LBs in PD (Sasaki et al., 2002) may be related to UPS failure. There is increasing evidence that UPS failure may underlie nigral degeneration and LB formation in both sporadic and familial PD (Dauer and Przedborski, 2003; Dawson and Dawson, 2003; Halliwell, 2002; McNaught et al., 2001; Valente et al., 2004). Recently, it has been shown that synthetic (Z-Ile-Glu(O*t*Bu)-Ala-Leu-al; PSI) and naturally occurring

(epoxomicin) inhibitors of proteasomal function can induce a progressive disorder in rats that closely recapitulates the features of PD (St et al., 2004). Our finding relating proteasomal impairment to increased clusterin expression is supported by the observation that in WI38 fibroblasts, epoxomicin exposure increases both sCLU and a cytoplasmic 64 kDa isoform of clusterin (Chondrogianni et al., 2003).

Increased clusterin expression has been associated with cell death responses in various tissues after toxic stress signals (Ahuja et al., 1994; Bursch et al., 1995; Buttyan et al., 1989; Danik et al., 1991; May, 1993; Sensibar et al., 1995; Tenniswood et al., 1992; Wong, 1994). Increased clusterin expression has been reported to be both cytoprotective (Criswell et al., 2003; Michel et al., 1997; Popper et al., 1997) and cytotoxic with a number of laboratories showing that cell death forms of clusterin are targeted to the nucleus (Caccamo et al., 2003; Caccamo et al., 2004; Lakins et al., 1998; Leskov et al., 2003; Yang et al., 2000).

There is no evidence that clusterin functions as an inducer of cell death in SH-SY5Y cells following lactacystin exposure. By RT-PCR, sCLU but not nCLU was detected in both control and lactacystin-treated cells. Western blot analysis following subcellular fractionation also failed to detect clusterin associated with the nucleus. The apparent molecular mass of clusterin (~40-45 kDa) and the association of clusterin with the ER suggest that in both control and lactacystin-treated cells sCLU is the major isoform expressed.

There is a growing body of evidence to suggest that stress-increased expression of sCLU is a cytoprotective effort (Criswell et al., 2003) and that sCLU exerts cytoprotection by functioning as a protein chaperone (Humphreys et al., 1999). sCLU is

capable of binding a wide range of partners (Wilson and Easterbrook-Smith, 2000) including partially folded stressed proteins (DeMattos et al., 2002; Poon et al., 2002) such as the major LB polypeptide component, α-synuclein (Zhou et al., 2004). The intracellular localization of LBs and the observation that *in vitro* astrocytes, but not neurons, secrete clusterin (Morgan et al., 1995; Pasinetti et al., 1994) suggest that in neurons clusterin exert its biological function intracellularly. Our finding that clusterin localizes to the ER, suggests that in neurons clusterin may possibly function as a stress-inducible ER protein chaperone.

It remains unclear how clusterin becomes associated with LBs. LB formation has been hypothesized to an aggresome-related event in response to increasing levels of abnomal proteins in neurons (McNaught et al., 2002). Aggresomes are structures that accumulate misfolded proteins thereby protecting against the deleterious spreading of misfolded proteins in the cell (Chung et al., 2001). Recently, it has been found that clusterin has strong aggresome forming capacity (Debure et al., 2003).

One possible model of the relationship between UPS failure, clusterin gene expression, LB formation and neurodegeneration in PD (Figure 4) is that in DA neurons clusterin expression is increased as a result of UPS failure caused by genetic and/or environmental insults. Functioning as a protective chaperone molecule, clusterin becomes associated with misfolded/damaged proteins that accumulate as a result of UPS failure thereby promoting cell survival. Over time, clusterin bound to its substrate becomes sequestered in within aggresomes and later LBs leading essentially to a loss of protective chaperone function and cell death.

In conclusion, the results of this study suggest that proteasome impairment contributes significantly to the upregulation of sCLU in SH-SY5Y cells supporting the hypothesis that the association of clusterin with LBs in PD may be related to UPS failure.

REFERENCES:

Ahuja HS, Tenniswood M, Lockshin R and Zakeri ZF. 1994. Expression of clusterin in cell differentiation and cell death. Biochem Cell Biol 72: 523-530.

Alves-Rodrigues A, Gregori L and Figueiredo-Pereira ME. 1998. Ubiquitin, cellular inclusions and their role in neurodegeneration. Trends Neurosci 21: 516-520.

Andersen JK. 2000. What causes the build-up of ubiquitin-containing inclusions in Parkinson's disease? Mech Ageing Dev 118: 15-22.

Bailey RW, Dunker AK, Brown CJ, Garner EC and Griswold MD. 2001. Clusterin, a binding protein with a molten globule-like region. Biochemistry 40: 11828-11840.

Bursch W, Gleeson T, Kleine L and Tenniswood M. 1995. Expression of clusterin (testosterone-repressed prostate message-2) mRNA during growth and regeneration of rat liver. Arch Toxicol 69: 253-258.

Buttyan R, Olsson CA, Pintar J, Chang C, Bandyk M, Ng PY and Sawczuk IS. 1989. Induction of the TRPM-2 gene in cells undergoing programmed death. Mol Cell Biol 9: 3473-3481.

Caccamo AE, Scaltriti M, Caporali A, D'Arca D, Scorcioni F, Astancolle S, **Mangiola M** and Bettuzzi S. 2004. Cell detachment and apoptosis induction of immortalized human prostate epithelial cells are associated with early accumulation of 45 KDa nuclear isoform of clusterin. Biochem J *in press*.

Caccamo AE, Scaltriti M, Caporali A, D'Arca D, Scorcioni F, Candiano G, **Mangiola M** and Bettuzzi S. 2003. Nuclear translocation of a clusterin isoform is associated with induction of anoikis in SV40-immortalized human prostate epithelial cells. Ann N Y Acad Sci 1010: 514-519.

Carrell RW and Lomas DA. 1997. Conformational disease. Lancet 350: 134-138.

Choi-Miura NH, Ihara Y, Fukuchi K, Takeda M, Nakano Y, Tobe T and Tomita M. 1992. SP-40,40 is a constituent of Alzheimer's amyloid. Acta Neuropathol (Berl) 83: 260-264.

Chondrogianni N, Stratford FL, Trougakos IP, Friguet B, Rivett AJ and Gonos ES. 2003. Central role of the proteasome in senescence and survival of human fibroblasts: induction of a senescence-like phenotype upon its inhibition and resistance to stress upon its activation. J Biol Chem 278: 28026-28037.

Chung KK, Dawson VL and Dawson TM. 2001. The role of the ubiquitin-proteasomal pathway in Parkinson's disease and other neurodegenerative disorders. Trends Neurosci 24: S7-14.

Clark AM and Griswold MD. 1997. Expression of clusterin/sulfated glycoprotein-2 under conditions of heat stress in rat Sertoli cells and a mouse Sertoli cell line. J Androl 18: 257-263.

Conn KJ, Gao WW, Ullman MD, McKeon-O'Malley C, Eisenhauer PB, Fine RE and Wells JM. 2001. Specific upregulation of GADD153/CHOP in MPP+-treated SH-SY5Y cells. J Neurosci Res 68: 755-760.

Conn KJ, Ullman MD, Larned MJ, Eisenhauer PB, Fine RE and Wells JM. 2003. cDNA microarray analysis of chagnes in gene expression associated with MPP+ toxicity in SH-SY5Y cells. Neurochem Res 28: 1873-1881.

Criswell T, Klokov D, Beman M, Lavik JP and Boothman DA. 2003. Repression of IR-inducible clusterin expression by the p53 tumor suppressor protein. Cancer Biol Ther 2: 372-380.

Danik M, Chabot JG, Mercier C, Benabid AL, Chauvin C, Quirion R and Suh M. 1991. Human gliomas and epileptic foci express high levels of a mRNA related to rat testicular sulfated glycoprotein 2, a purported marker of cell death. Proc Natl Acad Sci U S A 88: 8577-8581.

Dauer W and Przedborski S. 2003. Parkinson's disease: mechanisms and models. Neuron 39: 889-909.

Dawson TM and Dawson VL. 2003. Molecular pathways of neurodegeneration in Parkinson's disease. Science 302: 819-822.

Debure L, Vayssiere JL, Rincheval V, Loison F, Le DY and Michel D. 2003. **Intracellular clusterin causes juxtanuclear aggregate formation and mitochondrial alteration.** **J Cell Sci** 116: 3109-3121.

DeMattos RB, O'Dell MA, Parsadanian M, Taylor JW, Harmony JA, Bales KR, Paul SM, Aronow BJ and Holtzman DM. 2002. Clusterin promotes amyloid plaque formation and is critical for neuritic toxicity in a mouse model of Alzheimer's disease. **Proc Natl Acad Sci U S A** 99: 10843-10848.

Dickson DW. 2001. Alpha-synuclein and the Lewy body disorders. **Curr Opin Neurol 14**: 423-432.

Fenteany G and Schreiber SL. 1998. **Lactacystin, proteasome function, and cell fate.** J Biol Chem 273: 8545-8548.

Forno LS. 1996. Neuropathology of Parkinson's disease. J Neuropathol **Exp Neurol 55**: 259-272.

Halliwell B. 2002. Hypothesis: proteasomal dysfunction: a primary event in neurogeneration that leads to nitrative and oxidative stress and subsequent cell death. Ann N Y Acad Sci 962: 182-194.

Hershko A and Ciechanover A. 1998. The ubiquitin system. Annu Rev Biochem 67: 425-479.

Hochstrasser M. 1996. Ubiquitin-dependent protein degradation. Annu Rev Genet 30: 405-439.

Humphreys DT, Carver JA, Easterbrook-Smith SB and Wilson MR. 1999. Clusterin has chaperone-like activity similar to that of small heat shock proteins. J Biol Chem 274: 6875-6881.

Jones SE and Jomary C. 2002. Clusterin. Int J Biochem Cell Biol 34: 427-431.

Kimura K, Asami K and Yamamoto M. 1997. Effect of heat shock treatment on the production of variant testosterone-repressed prostate message-2 (TRPM-2) mRNA in culture cells. Cell Biochem Funct 15: 251-257.

Lakins J, Bennett SA, Chen JH, Arnold JM, Morrissey C, Wong P, O'Sullivan J and Tenniswood M. 1998. Clusterin biogenesis is altered during apoptosis in the regressing rat ventral prostate. J Biol Chem 273: 27887-27895.

Leskov KS, Klokov DY, Li J, Kinsella TJ and Boothman DA. 2003. Synthesis and functional analyses of nuclear clusterin, a cell death protein. J Biol Chem 278: 11590-11600.

Lidstrom AM, Bogdanovic N, Hesse C, Volkman I, Davidsson P and Blennow K. 1998. Clusterin (apolipoprotein J) protein levels are increased in hippocampus and in frontal cortex in Alzheimer's disease. Exp Neurol 154: 511-521.

May PC. 1993. Sulfated glycoprotein-2: an emerging molecular marker for neurodegeneration. Ann N Y Acad Sci 679: 235-244.

May PC and Finch CE. 1992. Sulfated glycoprotein 2: new relationships of this multifunctional protein to neurodegeneration. Trends Neurosci 15: 391-396.

McGeer PL, Kawamata T and Walker DG. 1992. Distribution of clusterin in Alzheimer brain tissue. Brain Res 579: 337-341.

McNaught KS, Olanow CW, Halliwell B, Isacson O and Jenner P. 2001. Failure of the ubiquitin-proteasome system in Parkinson's disease. Nat Rev Neurosci 2: 589-594.

McNaught KS, Shashidharan P, Perl DP, Jenner P and Olanow CW. 2002. Aggresome-related biogenesis of Lewy bodies. Eur J Neurosci 16: 2136-2148.

Michel D, Chatelain G, North S and Brun G. 1997a. Stress-induced transcription of the clusterin/apoJ gene. Biochem J 328 (Pt 1): 45-50.

Morgan TE, Laping NJ, Rozovsky I, Oda T, Hogan TH, Finch CE and Pasinetti GM. 1995. Clusterin expression by astrocytes is influenced by transforming growth factor beta 1 and heterotypic cell interactions. J Neuroimmunol 58: 101-110.

Oda T, Pasinetti GM, Osterburg HH, Anderson C, Johnson SA and Finch CE. 1994. Purification and characterization of brain clusterin. Biochem Biophys Res Commun 204: 1131-1136.

Pasinetti GM, Johnson SA, Oda T, Rozovsky I and Finch CE. 1994. Clusterin (SGP-2): a multifunctional glycoprotein with regional expression in astrocytes and neurons of the adult rat brain. J Comp Neurol 339: 387-400.

Poon S, Treweek TM, Wilson MR, Easterbrook-Smith SB and Carver JA . 2002. Clusterin is an extracellular chaperone that specifically interacts with slowly aggregating proteins on their off-folding pathway. FEBS Lett 513: 259-266.

Popper P, Farber DB, Micevych PE, Minoofar K and Bronstein JM. 1997. TRPM-2 expression and tunel staining in neurodegenerative diseases: studies in wobbler and rd mice. Exp Neurol 143: 246-254.

Rao RV, Hermel E, Castro-Obregon S, del Rio G, Ellerby LM, Ellerby HM and Bredesen DE. 2001. Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. J Biol Chem 276: 33869-33874.

Reddy KB, Karode MC, Harmony AK and Howe PH. 1996. Interaction of transforming growth factor beta receptors with apolipoprotein J/clusterin. Biochemistry 35: 309-314.

Rosenberg ME and Silkensen J. 1995. Clusterin: physiologic and pathophysiologic considerations. Int J Biochem Cell Biol 27: 633-645.

Rozen S and Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132: 365-386.

Sasaki K, Doh-ura K, Wakisaka Y and Iwaki T. 2002. Clusterin/apolipoprotein J is associated with cortical Lewy bodies: immunohistochemical study in cases with alpha-synucleinopathies. Acta Neuropathol (Berl) 104: 225-230.

Sensibar JA, Sutkowski DM, Raffo A, Buttyan R, Griswold MD, Sylvester SR, Kozlowski JM and Lee C. 1995. Prevention of cell death induced by tumor necrosis factor alpha in LNCaP cells by overexpression of sulfated glycoprotein-2 (clusterin). Cancer Res 55: 2431-2437.

Senut MC, Jazat F, Choi NH and Lamour Y. 1992. Protein SP40,40-like Immunoreactivity in the Rat Brain: Progressive Increase With Age. Eur J Neurosci 4: 917-928.

Sherman MY and Goldberg AL. 2001. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. Neuron 29: 15-32.

St PM, Perl DP, Brownell AL and Olanow CW. 2004. Systemic exposure to proteasome inhibitors causes a progressive model of Parkinson's disease. Ann Neurol 56: 149-162.

Tenniswood MP, Guenette RS, Lakins J, Mooibroek M, Wong P and Welsh JE. 1992. Active cell death in hormone-dependent tissues. Cancer Metastasis Rev 11: 197-220.

Valente EM, bou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del TD, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez-Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G and Wood NW. 2004. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 304: 1158-1160.

Voges D, Zwickl P and Baumeister W. 1999. The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu Rev Biochem 68: 1015-1068.

Wilson MR and Easterbrook-Smith SB. 2000. Clusterin is a secreted mammalian chaperone. Trends Biochem Sci 25: 95-98.

Wong P. 1994. Apoptosis, retinitis pigmentosa, and degeneration. Biochem Cell Biol 72: 489-498.

Yang CR, Leskov K, Hosley-Eberlein K, Criswell T, Pink JJ, Kinsella TJ and Boothman DA. 2000. Nuclear clusterin/XIP8, an x-ray-induced Ku70-binding protein that signals cell death. Proc Natl Acad Sci U S A 97: 5907-5912.

Yang CR, Yeh S, Leskov K, Odegaard E, Hsu HL, Chang C, Kinsella TJ, Chen DJ and Boothman DA. 1999. Isolation of Ku70-binding proteins (KUBs). Nucleic Acids Res 27: 2165-2174.

Zhou Y, Gu G, Goodlett DR, Zhang T, Pan C, Montine TJ, Montine KS, Aebersold RH and Zhang J. 2004. Analysis of alpha -synuclein-associated proteins by quantitative proteomics. J Biol Chem.

FIGURE LEGENDS:

Figure One- *Gene microarray analysis of lactacystin toxicity-* Shown are of images of plastic human 12K microarrays generated from cells treated for 24 hours with lactacystin (10 µM) or control conditions (vehicle). The arrows show differential gene expression of clusterin.

Figure Two- *RT-PCR and Western blot analyses of clusterin expression-* Cells were exposed to 10 µM lactacystin (L) or control (C) conditions for up to 24 hours prior to being harvested for RT-PCR and Western blot analyses. A. Electrophoretic separation of PCR products from a typical experiment is shown using RNA isolated from cells after incubation with (+) or without (-) lactacystin for 0.5, 2, 4, 8 16, and 24 hours and primers to G3PDH and clusterin B. Electrophoretic separation of PCR products from a typical

experiment is shown using RNA isolated 24 hours after exposure of cells to lactacystin (L) or control (C) conditions and primers to clusterin capable of discriminating sCLU (340 bp) from the alternatively spliced nuclear nCLU (220 bp) isoform. A 50 bp DNA ladder is shown to the left. **C.** Western blot analysis from a typical experiment is shown using total cell lysates isolated 24 hours after exposure of cells to lactacystin (L) or control (C) conditions and antibodies to clusterin (left panel). Coomassie staining of the gel after transfer (right panel) was performed to monitor equal loading of protein. Molecular weight standards in kilodaltons (kDa) are shown to the left of the blot. **D.** Western blot analysis was performed on enriched nuclear (N), endoplasmic reticular (ER) and soluble cytosol (S) fractions obtained from control (C) and lactacystin-treated (L) cells with antibodies to clusterin.

Figure Three - *Timecourse of clusterin gene expression, proteasomal activity, and cell viability in SH-SY5Y cells after exposure to 10 μM lactacystin-* Cells were exposed to lactacystin (L) or control conditions (C) for 0.5, 2, 4, 8, 16, 24, and 48 hours. At each timepoint, clusterin and G3PDH gene expression, cell viability, proteasomal activity, was quantified as described in the text. Data are graphed as % control where control represents those cells not treated with lactacystin. Time course represents the mean of quadruplicate values from a single experiment except for values with error bars where the data represents the mean ± SEM from 4 to 7 independent experiments.

Figure Four – *Model of the relationship between UPS failure, clusterin gene expression, LB formation and neurodegeneration in PD-* Clusterin expression is increased in DA

neurons as a result of UPS failure caused by genetic and/or environmental insults. Functioning as a protective chaperone molecule, clusterin becomes associated with misfolded/damaged proteins that accumulate as a result of UPS failure thereby promoting cell survival. Over time, clusterin bound to its substrate becomes sequestered in within aggresomes and later LBs leading essentially to a loss of protective chaperone function and cell death.
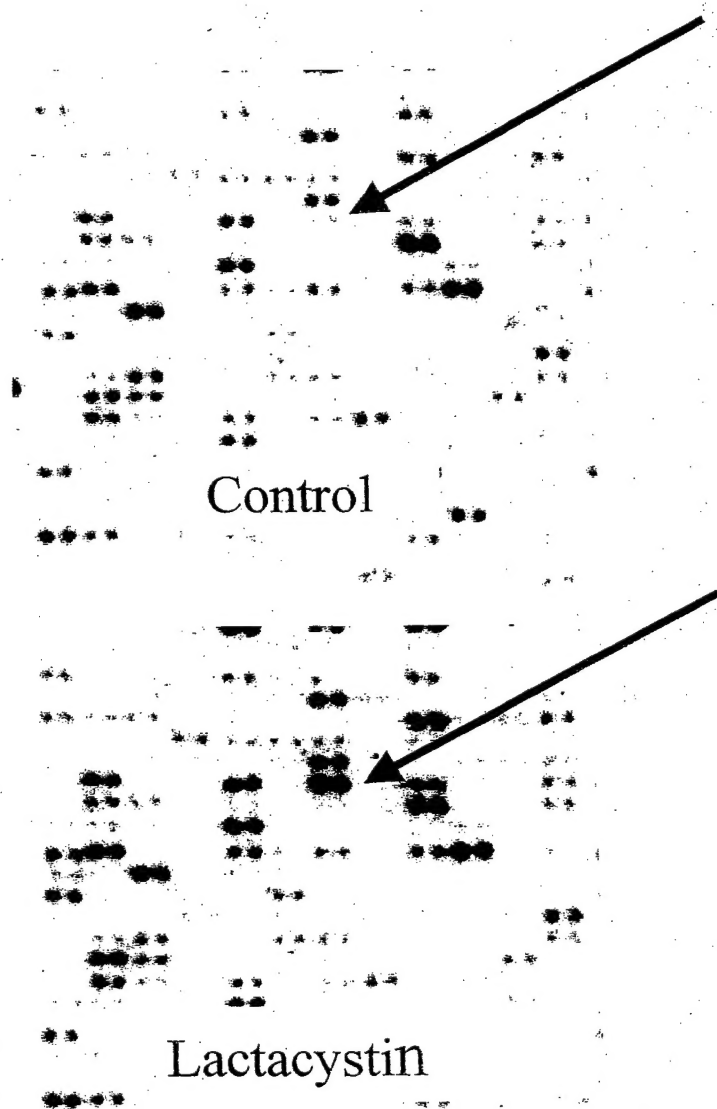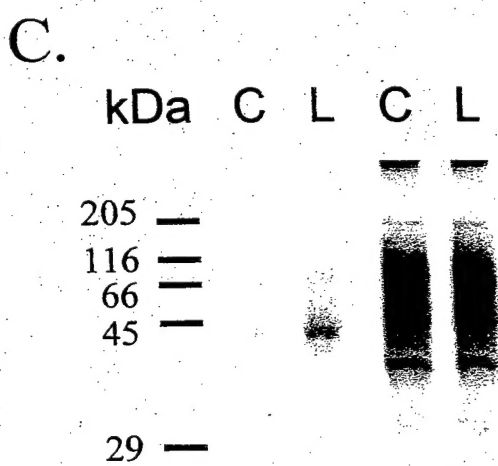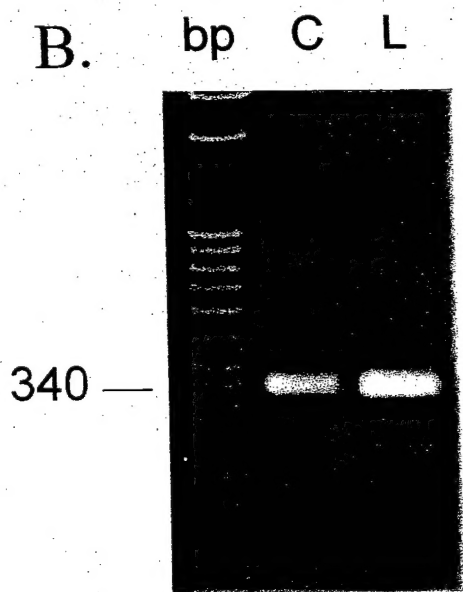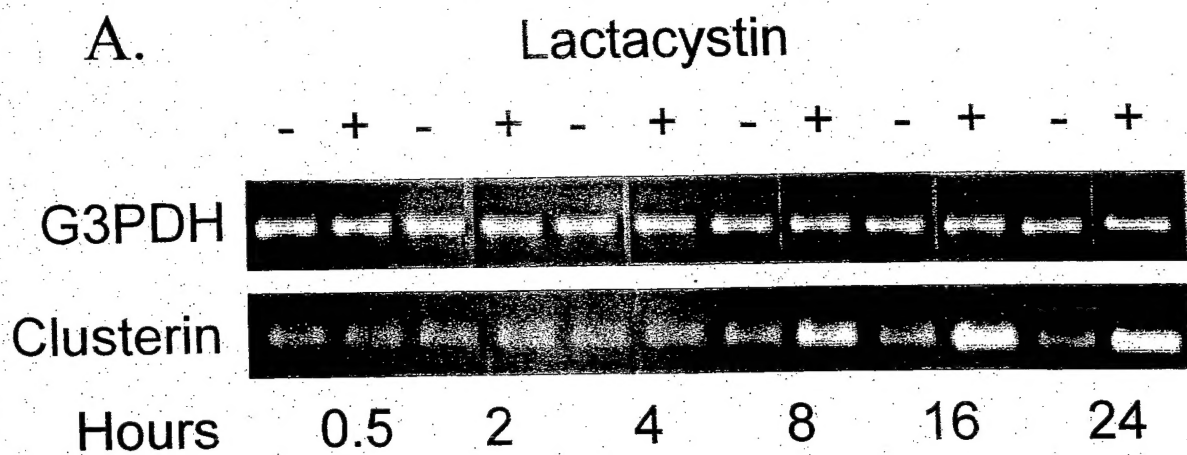
FIGURE ONE

Control

Lactacystin

A.

Lactacystin

| − | + | − | + | − | + | − | + | − | + | − | + |

G3PDH

Clusterin

Hours   0.5   2   4   8   16   24

B.

bp C L

340 —

C.

kDa C L C L

205 —
116 —
66 —
45 —

29 —

clusterin gel

D.

C L C L C L

N   ER   S

FIGURE THREE

FIGURE FOUR

Environmental insult   Genetic mutation

DOPAMINERGIC NEURONS

↓

UPS failure

↓

Increased sCLU expression
(protective chaperone molecule)

Sequestered in LBs
(loss of function)

Cell survival   Cell death